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Expression systems for adenovirus late proteins.

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Thesis submitted for the qualification of PhD

University of Warwick

Department of Biological Sciences

September 2000

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Declaration.

All work in this thesis was performed by the author unless otherwise stated. None of the material presented herein has at any time been presented for examination for any other degree.

Dedication.

For Mum, Dad, Tracey and Kirstie.

Thank you.

For E.N.H.

*Day dreaming
Chain smoking
Always laughing
Always joking
I remain the same
Did I tell you that I love you*

Summary.

During the past few decades a new approach has emerged for the treatment of human disease. In that short period, the concepts and techniques of gene therapy have progressed from being entirely fanciful to experimental clinical application. A major stumbling block for gene therapy is the inefficiency of gene transfer and the transient nature of therapeutic gene expression. Attempts to deliver therapeutic genes using replication-defective adenoviruses have been hampered by a strong host immune response to the vector, leading to clearance of transduced cells and loss of transgene expression. Current adenovirus vectors have an additional disadvantage of being able to carry only approximately 10 kb of exogenous DNA. The work here describes attempts to improve upon existing adenoviral vectors by addressing these limitations

In order to reduce the host immune response to the vector, additional deletions in the residual viral coding regions are required to prevent expression of immunogenic proteins. Deletion of the major late transcription unit (MLTU), which encodes virtually all of the viral structural proteins, would achieve this and would also increase the transgene carrying capacity of the vector. In order to create such a vector, a cell line would be required to complement the growth of the deleted vector by providing late gene functions *in trans*. The work presented here describes the successful cloning and subsequent analysis of the Ad5 MLTU with expression driven by the major late promoter (MLP). The expression plasmids constructed express one or more late proteins from each late gene segment in a transient assay. The plasmids also carry the EBNA-1 and oriP sequences from Epstein-Barr virus which allow the plasmids to be stably maintained in eukaryotic cells. Stable cell lines were constructed using these plasmids but no late protein expression from the MLTU could be detected. Attempts to activate expression from the major late promoter by providing viral transactivating factors *in trans* also proved to be ineffective.

To address these problems, an alternative inducible promoter was chosen. The sheep metallothionein Ia promoter was cloned upstream of the MLTU and this construct was then cloned into an episomal expression vector. This plasmid was also used successfully to regulate the expression of late proteins during transient transfection studies. However, a stable cell line constructed using the same plasmid did not show any expression of late proteins.

The reasons for the inability of any cell line constructed to express late proteins are still undetermined. Possible reasons discussed are plasmid rearrangement, promoter down-regulation and possible blocks to post-transcriptional processing and translation of the complex MLTU transcript. Suggested future studies include testing of these possibilities in order to gain further insight into the regulation of expression from the MLTU construct, ultimately leading to the construction of a cell line capable of complementing the growth of adenovirus vectors with late gene deficiencies.

Abbreviations.

α -1 AT:	α -1 antitrypsin
(p)TP:	(pre) terminal protein
A:	adenine
aa:	amino acids
A ₂₆₀ :	absorbance at 260 nm
Ab:	antibody
Ad:	adenovirus
Ad5:	adenovirus serotype 5
ADA:	adenosine deaminase deficiency
ADP:	adenovirus death protein
Adpol:	adenovirus DNA polymerase
Ag:	antigen
Amp:	ampicillin
ATP:	adenosine triphosphate
β -gal:	β -galactosidase
bp:	base pair
BSA:	bovine serum albumin
C:	cytosine
cDNA:	complementary deoxyribonucleic acid
CF:	cystic fibrosis
CFTR:	cystic fibrosis transmembrane regulator
Ci:	Curie
CIAP:	calf intestinal alkaline phosphatase
CMV:	cytomegalovirus
CPE:	cytopathic effect
CsCl:	cesium chloride
CTL:	cytotoxic T-lymphocyte
DBP:	DNA binding protein
dl:	deletion
DMD:	Duchenne muscular dystrophy
DMEM:	Dulbecco's modified Eagle's medium

DMSO:	dimethyl sulphoxide
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphate
ds:	double-stranded
DTT:	dithiothreitol
EBNA-1:	Epstein-Barr virus nuclear antigen 1
EBV:	Epstein-Barr virus
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme linked immunosorbent assay
FCS:	foetal calf serum
g:	gram
G:	guanine
Hepes:	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV:	human immunodeficiency virus
HRP:	horseradish peroxidase
HSV:	herpes simplex virus
IAA:	isoamyl alcohol
Ig:	immunoglobulin
IL-2 (3,4,5 etc.):	interleukin 2 (3,4,5 etc.)
IPTG:	isopropyl-1-thio- β -D-galactosidase
ITR:	inverted terminal repeat
IVa2:	polypeptide IVa2
kb:	kilobase
kbp:	kilobase pair
kDa:	kilodaltons
LB:	Luria-Bertani
M:	molar
MHC:	major histocompatibility complex
MLP:	major late promoter
MLTU:	major late transcription unit
moi:	multiplicity of infection
MOPS:	3-[N-Morpholino]propanesulphonic acid
mRNA:	messenger ribonucleic acid

MW:	molecular weight
NCS:	newborn calf serum
NF I, II:	nuclear factor I, II
nm:	nanometer
NP40:	Nonidet P40
ORF:	open reading frame
OTC:	ornithine transcarbamylase
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
RNA:	ribonucleic acid
RNase:	ribonuclease
rpm:	revolutions per minute
RSV:	respiratory syncytial virus
S:	Svedberg unit
SCID:	severe combined immunodeficiency
SDS:	sodium dodecyl sulphate
ss:	single stranded
T:	thymine
TEMED:	N,N,N',N'-tetramethyl-ethylenediamine
Tris:	tris (hydroxymethyl) aminomethane
UV:	ultraviolet
V:	volts
wt:	wild type
x g:	times gravitational acceleration

Chapter 1 : Introduction.

Chapter 1 : Introduction.

1.1. Introduction.

A conceptually new approach to the treatment of human disease has emerged during the past two decades, termed gene therapy, which promises to revolutionise modern medicine. Unfortunately gene therapy has an Achilles heel: inefficient gene delivery.

Viruses have evolved very effective methods of delivering their own nucleic acids to cells, thereby allowing their own replication and propagation. For this reason several viruses have been investigated for use as gene delivery vehicles, with mixed results.

The aim of this introduction is to give a brief overview of the fundamentals of gene therapy, followed by a detailed review of the gene-delivery vehicle that is the subject of the research reported in this thesis, the adenovirus vector.

1.2. Gene therapy – a brief history.

Gene therapy, broadly defined as the treatment or prevention of disease by gene transfer, is regarded by many as a potential revolution in medicine. This is because gene therapies aim to treat or eliminate the underlying cause of disease, whereas most current therapies merely alleviate the symptoms. Although originally intended as a treatment for inherited disorders (Friedmann and Roblin, 1972), gene therapy is now being applied to many other fields of medicine, particularly the treatment of cancer and infectious disease (reviewed by Weatherall, 1995).

Gene therapy is generally considered to be a relatively new form of therapy, largely because it is only over the past decade that it has been widely recognised as a valid approach to treating disease (reviewed by Friedmann, 1992). Despite the apparent infancy of gene therapy, the underlying principals can be traced back to the classical studies of Avery, McLeod and McCarty (1944) into DNA-mediated genetic transformation in pneumococcus. Subsequent experiments established that mammalian cells could be altered by the introduction of exogenous DNA (Kay,

1961). These findings, in conjunction with the discovery of mammalian cells stably transformed by DNA introduced by papovaviruses (Sambrook *et al.*, 1968), led to the proposal that viruses could be used as vectors to deliver therapeutic DNA into cells (Rogers and Pfunderer, 1968) and mark the birth of gene therapy.

Although the term gene therapy was coined almost thirty years ago, it was not until the advent of techniques for the efficient isolation and manipulation of DNA in the 1970s that it began to become a feasible proposition. The subsequent advances in biotechnological expertise and increased understanding of many disease states at the molecular level have led to an expansion in gene therapy research. This growing interest led to the first human gene therapy clinical trial for treatment of adenosine-deaminase deficiency (ADA), commencing in 1990 (Blaese *et al.*, 1995), and marked the transformation of gene therapy from a human geneticist's pipedream, to the focused reality of a whole new industry.

1.2.1. Applications and candidate diseases.

Ideally, gene therapy would emulate transplant surgery, removing a mutant gene and replacing it with a normal copy. However, this approach has many technical difficulties and current research in gene therapy is directed towards gene augmentation. This involves introducing a gene into the target cell in a way that will allow it to produce sufficient of its product to compensate for lack of expression of its defective counterpart. Broadly speaking, there are two approaches to gene therapy; the *ex vivo* and *in vivo* approaches. In the *ex vivo* approach, cells are removed from the patient, transfected with the therapeutic gene and then reintroduced. This has the advantage of efficient and controllable gene transfer but has the drawback of being-patient specific. The *in vivo* approach involves the direct administration of the gene transfer vector to the patient. This is therefore not patient-specific but does have the disadvantage that cells are more difficult to transfect *in vivo*.

There are a number of diseases, both inherited and acquired, which are candidates for treatment by gene therapy. Although originally envisioned as a treatment for monogenic recessive disorders, today gene therapy is being applied to other areas such as cancer therapy and infectious diseases (reviewed by Verma and Somia, 1997; Mountain, 2000). Approximately 300 clinical protocols have been approved to date, with two thirds of them directed at cancer, and most of the remainder at monogenic

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disorders and infectious diseases (Table 1.1). It has been widely publicised that the outcome of clinical trials in terms of gene expression and clinical benefit has been disappointing. This is largely due to shortcomings in vector technology, but as vector technology progresses clinical benefits from gene therapy should become more apparent. In fact, within the past year, clinical benefit from gene therapy has been demonstrated for the first time. Patients receiving treatment for critical limb ischaemia were given naked DNA injections (section 1.2.2.2.1.) of plasmid encoding vascular endothelial growth factor (VEGF) into skeletal muscle. A dramatic long lasting effect was observed in many patients, including those who would otherwise have faced amputation (Baumgartner *et al.*, 1998; 2000). In addition, positive results have been reported in a trial for the treatment of severe combined immunodeficiency disorder (SCID) – XI disease (Cavazzana *et al.*, 2000). SCID-XI is an X-linked inherited disorder characterised by a block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations in the gene encoding the common cytokine receptor gamma chain (gammac). The gene therapy trial involved the *ex vivo* delivery of gammac cDNA to CD34⁺ cells using a Moloney retrovirus derived-vector. After a 10-month follow-up period, gammac transgene-expressing T and NK cells were detected in two patients. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of a disease phenotype and hence, clinical benefit.

Table 1.1: Distribution of gene-transfer clinical trials in North America and Europe (June 1999).

Disease.	Number of protocols.
Cancer.	216
Monogenic diseases.	49
Infectious diseases.	24
Cardiovascular diseases.	8
Rheumatoid arthritis.	2
Cubital tunnel syndrome.	1
Total number of protocols.	300
<i>In vivo</i> gene-transfer.	144
<i>Ex vivo</i> gene-transfer.	156

Adapted from Mountain, 2000.

1.2.2. Gene delivery systems.

Gene delivery vectors fall into three main types: physical, non-viral and viral. Physical methods include the use of needle-free injectors and electroporators. Non-viral approaches involve the use of naked DNA or DNA complexed with cationic lipids (liposomes) or cationic polymers. Many different viruses are being adapted as vectors, but the most advanced in their development are retrovirus, adenovirus and adeno-associated virus (AAV) vectors. Each method has its own set of advantages and disadvantages (Table 1.2) so the choice of vector will often be dictated by the properties required for a specific application. For example if only short-lived transgene expression is required, then the adenoviral vectors are ideal. Whereas if long term expression is required, an integrating vector such as AAV or a lentivirus would be more appropriate. All current DNA delivery methods have limitations and an ideal vector would have to combine properties from both viral and synthetic systems. The properties of this ideal vector would include production at high concentration, site-specific integration or episomal maintenance, transgene expression regulation, the ability to target specific cell types and low immunogenicity. No single vector currently fulfills all these requirements, in fact it is largely due to the

Table 1.2 : Advantages and disadvantages of the most common gene-transfer vectors.

	Advantages.	Disadvantages.
Adenovirus	Very high transduction efficiency <i>in vivo</i> and <i>ex vivo</i> . Transduces proliferating and non-proliferating cells. High titres of recombinants easily produced.	Inflammatory response limits transgene expression duration and repeat administration. Insert size limit of 11 kbp (unless grown with helper virus).
Retrovirus	High transduction efficiency <i>ex vivo</i> . Pre-existing immunity unlikely. Recombinants easily produced. Substantial clinical experience <i>ex vivo</i> .	Low transduction efficiency <i>in vivo</i> . Insert size limit of 8 kbp. Risk of insertional mutagenesis. Transduces only proliferating cells.
Lentivirus	Transduces proliferating and non-proliferating cells. Transduces haematopoietic stem cells.	Safety concerns from immunodeficiency virus origins. Insert size limit of 8 kbp.
AAV	Transduces a wide variety of cells <i>in vivo</i> . Prolonged expression after genome integration. Low immunogenicity.	Insert size limit of 4.5 kbp. Requires helper virus for growth. Little clinical experience. Concerns over insertional mutagenesis.
Herpesvirus	Neurotropic. Large insert size of 50 kb.	Inflammatory response. Genome difficult to manipulate <i>in vitro</i> .
Naked DNA	Low immunogenicity. Good safety profile. No insert size limit. Clinical efficacy demonstrated.	Short duration of transgene expression. Very inefficient transfection <i>ex vivo</i> and <i>in vivo</i> .
Condensed DNA particles	Efficient transfection <i>ex vivo</i> . Low immunogenicity. Good safety profile. No insert size limit. Retargeted transfection possible.	Inefficient transfection <i>in vivo</i> . Very short duration of transgene expression. No clinical experience.
Cationic lipids	Efficient transfection <i>ex vivo</i> . Low immunogenicity. Good safety profile. No insert size limit.	Inefficient transfection <i>in vivo</i> . Very short duration of transgene expression. Retargeting transfection difficult.

constructs within mouse skeletal muscle cells. The DNA persisted for at least 60 days, although the mRNA and expressed protein had a half-life of less than 24 hours.

The method of direct DNA injection is a simple, inexpensive and relatively non-toxic procedure. However, it does have limitations, mainly low transfection efficiency, transient gene expression that is localised only at the site of injection and unsuitability for targeting. It may have potential as a vaccination procedure due to the presence of immunostimulatory sequences in the plasmid DNA (Krieg *et al.*, 1995). CpG dinucleotide sequences, cytosine followed by guanine elicit immune responses in animals and are regarded as a very powerful adjuvant, beneficial for any vaccination procedure.

1.2.2.2.2. Cationic lipids.

Monocationic lipids form liposomes that spontaneously bind polyanionic DNA or RNA. These can then absorb to the cell membrane and deliver the nucleic acid directly into the cytoplasm, bypassing the endosomal pathway. Several lipid-DNA complexes have been shown to give reasonable transfection efficiency *ex vivo*, and *in vivo* after administration to the airway epithelium. Unfortunately transgene expression is transient and transfection cannot be targeted to a particular cell type (reviewed by Li and Huang, 2000; Mountain, 2000).

1.2.2.2.3. Condensed DNA particles.

Another class of synthetic vector that has been extensively studied is based on cationic polymers (reviewed by Li and Huang, 2000; Mountain, 2000). These polymers, typically polylysine or polyethyleneimine, condense DNA by electrostatic interaction into small particles, protecting it from degradation and enhancing uptake. The advantage of this method is that the particles can be retargeted by addition of ligands for cellular receptors, although transfection efficiency is low.

1.2.2.3. Viral delivery systems.

1.2.2.3.1. Retrovirus vectors.

Retrovirus vectors constitute the most widely used vectors to date, having been shown to be safe and effective at delivering transgenes in *ex vivo* applications. Over half of the clinical trials approved in North America and Europe have involved the use of a retroviral vector (reviewed by Verma and Somia; 1997, Mountain, 2000). The majority of retroviral vectors are based on murine leukaemia viruses (MLVs); these are made replication-deficient by replacing the viral genes, *gag*, *pol* and *env*, with a transgene expression cassette. The lack of these genes is compensated for by growth in a packaging cell line that expresses the deleted viral genes.

A major advantage of retrovirus vectors is that they integrate a DNA copy of their RNA genome into the target cell's genome. This leads to constant levels of gene expression *in vitro* and all daughter cells inherit a copy of the transgene. However, when the cells are returned *in vivo* they often lose expression of the foreign gene within a few weeks. This silencing phenomenon is not well understood, but is likely to be the result of methylation in the vicinity of the promoter. Although advantageous this integration mechanism is random which could lead to a risk of insertional mutagenesis. This could result in the activation of oncogenes or deactivation of tumour suppressor genes. Although the theoretical probability of such an event is quite low, it is still of some concern. Another limitation of retroviral vectors is their inability to infect non-dividing cells, as they require breakdown of the nuclear envelope during mitosis before the genome can integrate. However, this is not true for retroviral vectors based on lentiviruses such as HIV and SIV, which have been shown to effectively transfect post-mitotic and quiescent cells (reviewed by Trono, 2000).

1.2.2.3.2. Adeno-associated virus (AAV) vectors.

AAV is a member of the *Parvoviridae* family and normally requires coinfection with a helper virus (such as adenovirus or herpes simplex virus) for lytic replication in cell culture (reviewed by Flotte and Carter, 1995). As for retroviruses, AAV is also capable of integrating into the target cell chromosome, with wild type AAV specifically integrating into one site on human chromosome 19. It appears however

that recombinant AAV vectors integrate much less efficiently and in a random manner (reviewed by Monahan and Samulski, 2000; Mountain, 2000). A major drawback to using AAV as a gene transfer vector is the limiting size of its genome, which can only accommodate up to 4.5 kb of foreign DNA. Conversely, its genome is very simple and easily manipulated *in vitro*. The difficult manufacturing process imposes other limitations on the use of AAV vectors. The required use of a helper virus presents problems of low titre, contamination and costly purification procedures. Only a few clinical trials involving AAV have been initiated to date, but preliminary results suggest that long term expression is feasible (Wagner, 1998). With the recent developments allowing the production of high-titre AAV stocks free of helper virus contamination (reviewed by Monahan and Samulski, 2000), and AAV's ability to transduce cells both *in vivo* and *in vitro*, more AAV-based therapies are likely to reach the clinic in the near future.

1.2.2.3.3. Herpesvirus vectors.

Many different herpesviruses may have potential as gene delivery vectors, but exploitation of this potential has, to date, only been explored using herpes simplex virus (HSV) (reviewed by Efsthathiou and Minson, 1995). HSV is a DNA virus with a genome of approximately 150 kbp, it is naturally neurotropic and can establish latency in neurons in a manner which does not depend on viral replication (reviewed by Roizman and Sears, 1996). This makes it an ideal candidate to deliver genes to the central nervous system, in order to treat conditions such as Parkinson's disease. HSV can be rendered replication-defective by deleting essential gene functions; growth of these vectors in complementing cell lines allows incorporation of about 50 kbp of foreign DNA. There have been problems associated with inflammatory reactions in the recipients of these vectors (Wood *et al.*, 1994), although recently derived vectors have addressed these problems by introduction of further attenuating mutations (Oligino *et al.*, 1998). HSV can also form the basis of artificial amplicons, plasmids which contain the viral origin of replication and packaging signal (Frenkel *et al.*, 1994). These are packaged into virus particles in the presence of a replication-defective helper virus, thus increasing the insert-size limit to almost 150 kbp. The use of these amplicons has limitations in that they only grow to low titres and contamination of the vector stock with helper virus can be a problem.

1.2.2.3.4. Adenovirus vectors.

Retroviruses aside, vectors based on adenovirus are the most commonly used gene delivery vehicles in clinical trials today. This is the virus used in the studies presented here and, as such, warrants a more detailed review. Accordingly, before reviewing the progress and advances in adapting this virus for use in gene therapy protocols, an account will be given of this virus, its pathogenesis and replication cycle.

1.3. The adenoviruses.

1.3.1. Classification.

Adenoviruses were first isolated in the winter–spring of 1952-53 by two groups of investigators working independently. In 1953 Rowe and colleagues observed degeneration of primary cell cultures derived from human adenoids, the agent responsible being termed the adenoid degenerating (AD) agent (Rowe *et al.*, 1953). During the same winter another group, studying an epidemic of respiratory illness in military recruits, isolated an agent able to cause degenerative changes in cultures of HeLa cells. This was designated the respiratory illness (RI) agent and was soon shown to be related to the AD agent by complement fixation and neutralisation tests (Hilleman and Werner, 1954). In 1956 the name “adenovirus” was given to these agents, after the original source of tissue in which the viral prototype strain was isolated (Enders *et al.*, 1956).

Since the isolation of the prototype virus, over 50 serotypes of adenoviruses have been isolated based on their differential resistance to neutralisation by antisera to other adenoviruses. These constitute the virus family *Adenoviridae*, which is divided into two genera, *Mastadenoviridae* and *Aviadenoviridae*. The *Aviadenoviridae* are viruses of birds while the *Mastadenoviridae* infect mammals, and includes human, simian, bovine, porcine, ovine, equine, canine and opossum viruses (reviewed by Shenk, 1996). The human adenoviruses have been divided into six subgroups, A-F, based on their ability to agglutinate red blood cells from different sources; thus haemagglutination is inhibited by antisera specific for viruses of the same subgroup. The virus used as the basis for the studies presented here was human adenovirus, subgroup C, serotype 5 (Ad5). Together with serotype 2, these are the two serotypes that have been studied in most detail. The sequences of the two genomes are 94.7 % identical and their gene organisation is virtually the same. Because of this similarity, data derived from studying one of these viruses is generally assumed to apply to both.

1.3.2. Pathogenesis of disease.

Human adenoviruses cause a wide range of diseases in their hosts, most commonly infections of the upper respiratory tract (Ad1,2,3,5 and 6). These infections are usually self-limiting, but can lead to pneumonia and some epidemics of Ad7 have

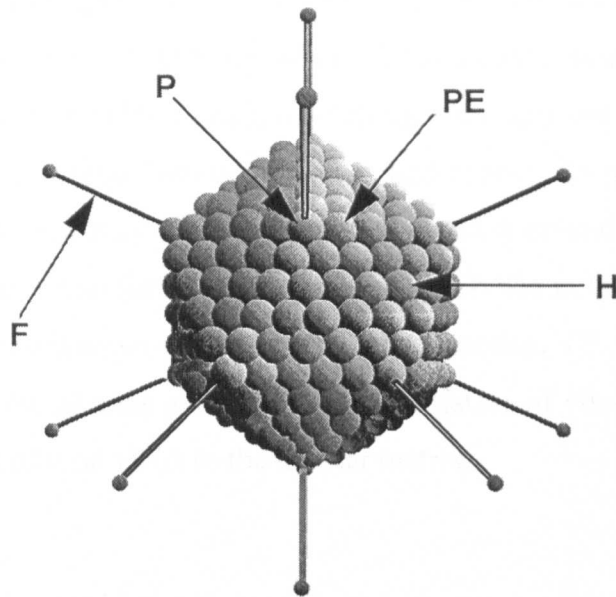
led to considerable mortality. Adenoviruses can also infect and replicate at other sites, leading to acute follicular conjunctivitis (Ad3 and 7), acute haemorrhagic cystitis (Ad11 and 21), gastroenteritis (Ad40 and 41) and occasionally meningoencephalitis. Many adenovirus infections are subclinical and result in antibody formation that is probably protective against exogenous reintroduction of the same serotype. Adenoviruses can achieve persistence in some tissues (tonsils, adenoids and intestine) of an infected host, although the mechanism of persistence for adenoviruses is poorly understood. All human adenoviruses tested are capable of transforming rodent cells *in vitro* and some are highly oncogenic in rodents (Ad12, 18 and 31). However, neither Ad5 nor any other group C adenovirus is known to be tumourigenic in humans (reviewed by Shenk, 1996).

1.3.3. Virion structure.

Adenoviruses are nonenveloped DNA-protein complexes of icosahedral symmetry (20 triangular faces and 12 vertices) with a diameter of 70-100 nm. The double stranded (ds) DNA genome (35938 bp for Ad5; Chroboczek *et al.*, 1992) constitutes 13 % of the mass of the virion, the remaining 87 % being protein with trace amounts of carbohydrate due to glycosylation of the fibre protein (reviewed by Shenk, 1996). The protein shell (capsid) comprises 252 subunits (capsomeres), of which 240 are hexons (so called because they are each surrounded by six neighbouring capsomeres) forming the faces and edges of triangular facets. The remaining 12 subunits are pentons (having five neighbouring capsomeres) which form the vertex capsomeres. Each penton contains a base unit within the body of the capsid and a projecting fibre, which terminates in a knob (reviewed by Stewart *et al.*, 1993). The structure and polypeptide components of the adenovirus capsid are depicted in figure 1.1a.

The protein capsid is composed of at least 11 polypeptides (figure 1.1b) as determined by SDS-PAGE and open reading frame (ORF) analysis, many of which are encoded by the major late transcription unit (MLTU). The capsid of the virion is composed of seven known polypeptides. Three tightly associated molecules of polypeptide II, the most abundant virion constituent, form each hexon capsomere. Associated with these hexons are polypeptides VI, VIII and IX, which serve to stabilise the capsomere framework, with polypeptides VI and VIII probably bridging between the capsid and core components. The penton base protein is composed of five

A.



B.

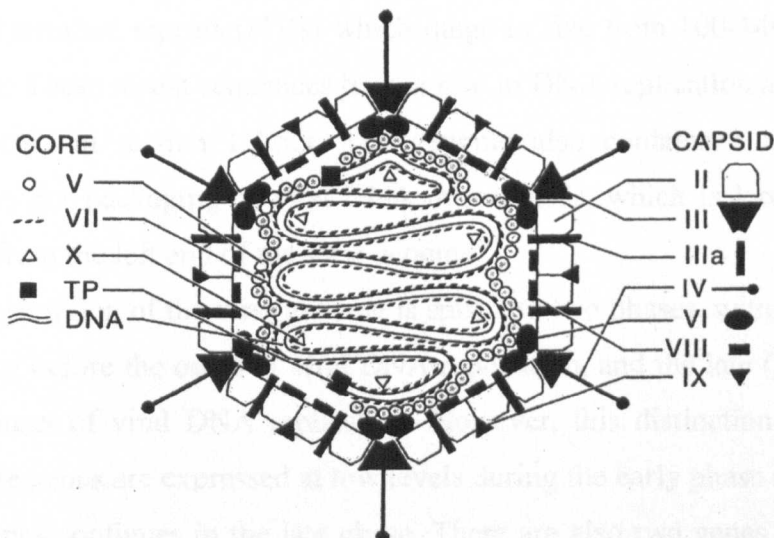


Figure 1.1. Models of the adenovirus virion. **A:** A three-dimensional representation of the adenovirus particle. Indicated are hexons (H), peripentonal hexons (PE) pentons (P), and fibre (F). **B:** A stylised cross-section of the adenovirus particle based on current understanding of its polypeptide components. Virion constituents are designated by their polypeptide number, as noted in the text, with the exception of terminal protein (TP). From Stewart *et al.* (1991).

molecules of polypeptide III and has the fibre protein (a trimer of polypeptide IV) projecting from within it. The core of the virion contains four polypeptides, V, VII, μ and terminal protein (TP), associated with the viral genome. Polypeptide V binds to pentons and may bridge between the core and capsid. Polypeptide VII is the major core constituent and may act as a histone-like centre around which the viral DNA is wrapped and may also form links with polypeptide IIIa in the capsid. The function of the μ protein is unknown whilst the remaining protein, TP, is covalently attached to the 5' ends of the genome and serves in the initiation of viral DNA replication and in the attachment of viral DNA to the nuclear matrix.

1.3.4. Genome organisation.

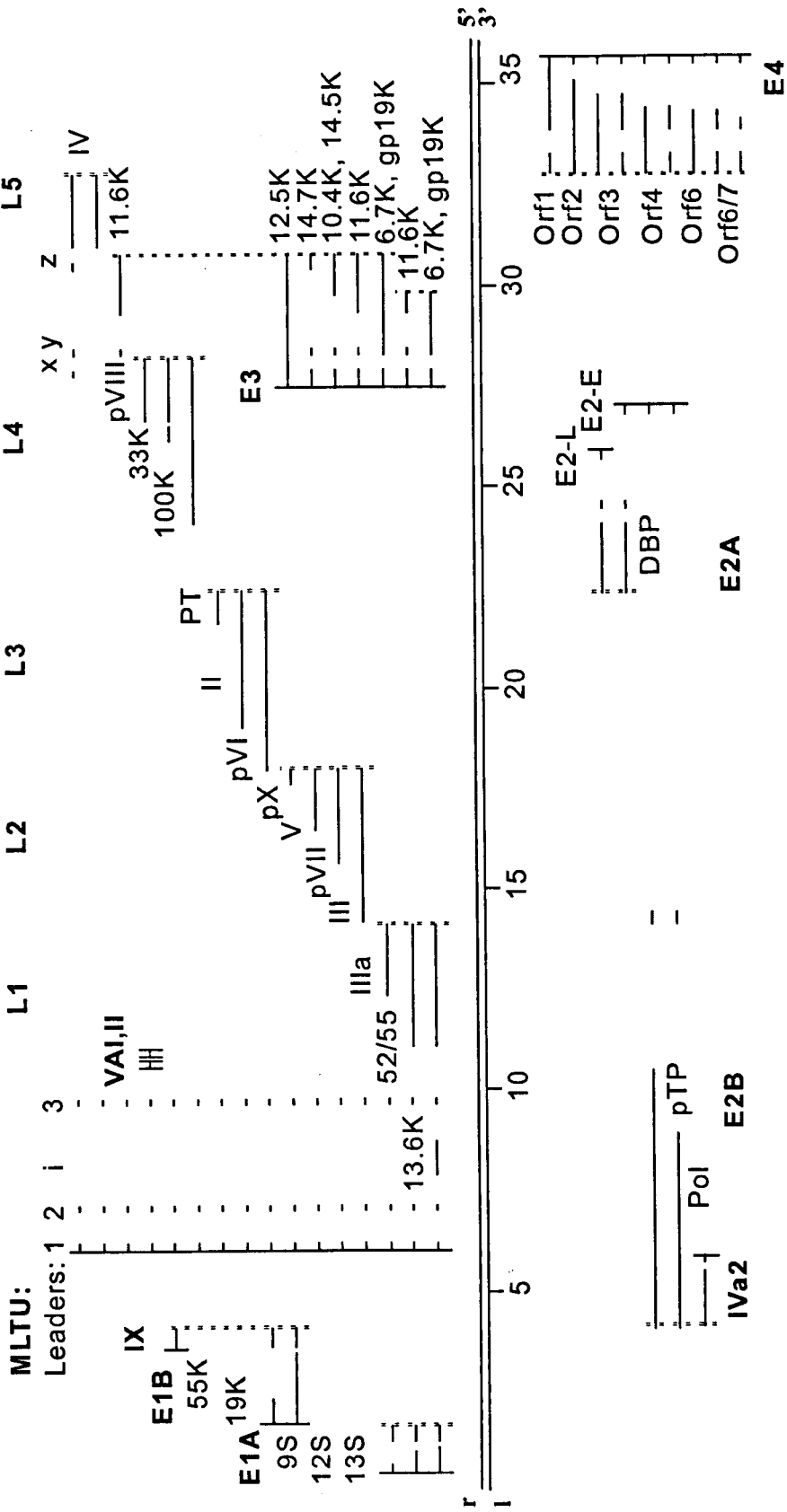
The adenovirus genome is a linear double stranded DNA molecule, with short inverted terminal repeats (ITRs) which range in size from 100-140 bp, depending on serotype. These repeat sequences have a role in DNA replication and are discussed in more detail in section 1.3.5.3. The genome also contains a *cis*-acting sequence necessary for packaging of viral DNA into capsids, which is located approximately 260 bp from the left end of the viral genome.

Transcription of the viral genome is split into two phases, with the early (E) phase occurring before the onset of viral DNA replication, and the late (L) phase beginning at the onset of viral DNA replication. However, this distinction is not absolute as some late genes are expressed at low levels during the early phase and transcription of early genes continues in the late phase. There are also two genes (IVa2 and IX) that are transcribed at an intermediate stage and are termed delayed early (reviewed by Shenk, 1996). The transcription map of Ad5 is shown in figure 1.2. The program of gene expression starts from both strands of the viral genome, the rightward (r) strand encodes E1, E3, IX, viral associated (VA) RNA and MLTU, whilst the leftward (l) strand encodes E2, E4 and IVa2. All genes are transcribed by cellular RNA polymerase II with the exception of VA RNA genes that are transcribed by RNA polymerase III. Transcription starts from both ends of the genome; thus the first genes expressed are E1A and E4. Each gene transcribed gives rise to multiple RNA species that are differentiated by alternative RNA splicing and in some cases by the use of different poly (A) sites. An example of this is the MLTU that produces at least 18 distinct mRNAs grouped into five families, termed L1 – L5. Indeed it was the analysis

Figure 1.2. Human adenovirus type 5 transcription map.

Adapted from Leppard (1998).

The virus genome is represented in the centre by parallel lines, with numbering indicating the distance from the conventional left end in kb. Genes or gene regions are shown in boldface. mRNAs are shown as solid lines, with gaps to indicate introns; RNA polymerase II promoters are shown as solid vertical lines, with polyadenylation sites designated by broken vertical lines. RNA polymerase III transcripts are shown as paired vertical lines. mRNAs transcribed from the r-strand are shown above the genome, those transcribed from the l-strand are shown below. Proteins translated from mRNAs are indicated adjacent to the mRNA; conventions for naming proteins are given in section 1.3.4. Abbreviations: PT, virion protease; DBP, DNA binding protein; pTP, terminal protein precursor; Pol, DNA polymerase.



of adenovirus mRNA which led to the discovery of RNA splicing (Berget *et al.*, 1977; Chow *et al.*, 1977). Proteins produced by each transcription unit are shown in figure 1.2. These are named either by sedimentation coefficients of the encoding mRNA (e.g. E1A-13S), molecular weight (e.g. E1B-55K), the open reading frame from which they are expressed (e.g. E4-Orf6) or function (e.g. DNA binding protein). Late structural proteins are numbered II – IX in order of decreasing size (reviewed by Shenk, 1996).

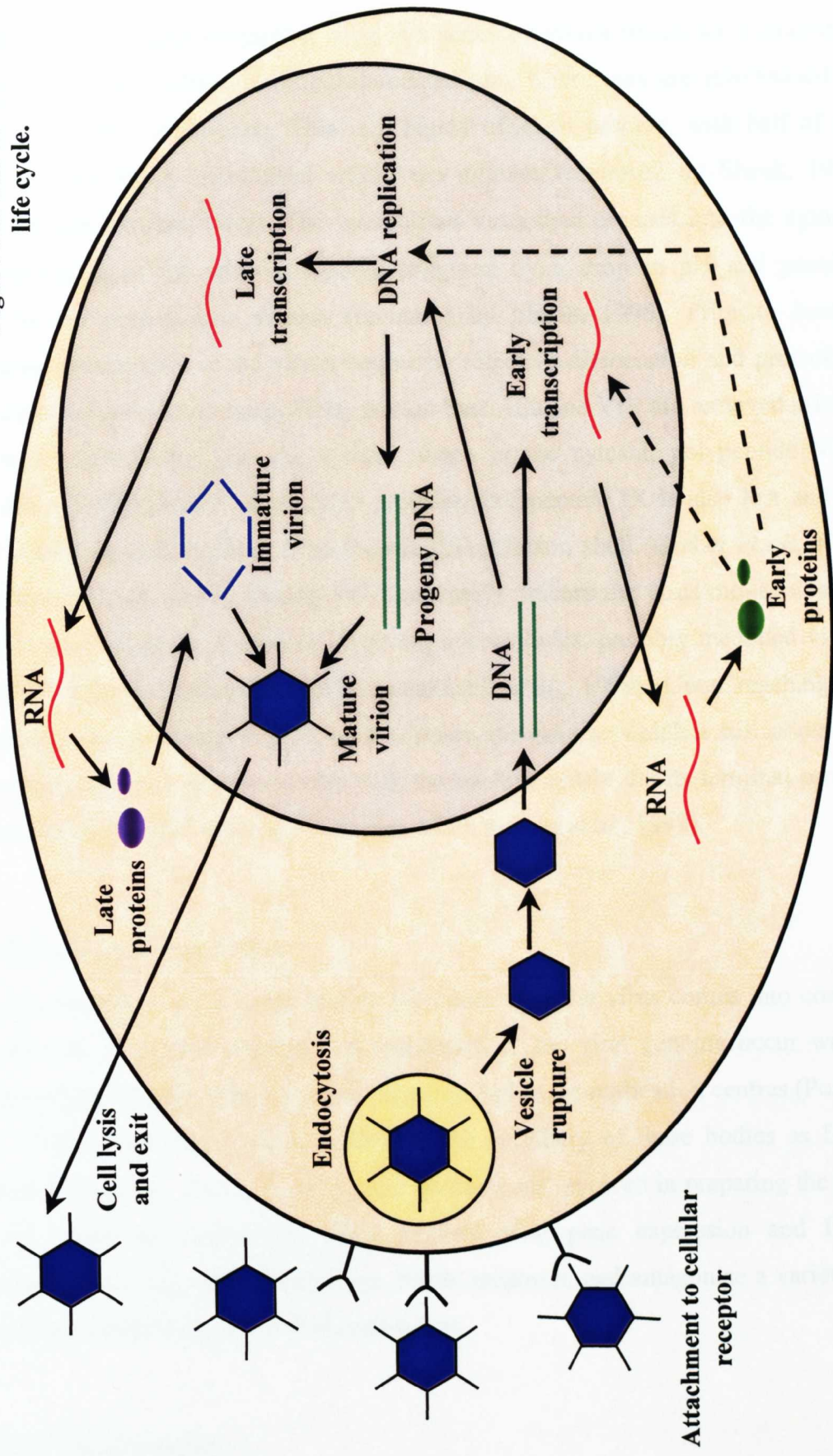
1.3.5. Replicative cycle.

A simplified adenovirus replication cycle is presented diagrammatically in figure 1.3.

1.3.5.1. Adsorption and entry.

The first step in human adenovirus infection consists of virus-cell recognition and attachment involving the carboxy-terminal region of the viral fibre protein (which forms the terminal knob of the fibre trimer) and host cell surface receptors. Two fibre receptors have been described to date, a 46 kDa glycoprotein termed the coxsackievirus – adenovirus receptor (CAR) (Bergelson *et al.*, 1997; Tomko *et al.*, 1997) and the major histocompatibility complex class I (MHC-I) $\alpha 2$ domain (Hong *et al.*, 1997). CAR protein can function as a cellular attachment protein for serotypes from subgroups A, C, D, E and F (Roelvink *et al.*, 1998) and recent findings suggest CAR is the primary receptor, with MHC-I $\alpha 2$ domain only being used in the absence of CAR (Davison *et al.*, 1999). Detailed analysis of the CAR protein has revealed that the fibre binding activity is located in the amino-terminal IgV-related extracellular domain and only this domain is required for virus attachment and entry (Bewley *et al.*, 1999; Freimuth *et al.*, 1999; Roelvink *et al.*, 1999; Wang and Bergelson, 1999; Kirby *et al.*, 2000). Subsequent efficient internalisation of adenovirus into cells requires a second interaction, mediated by the viral penton base and another cell surface protein. These cellular proteins are members of the integrin family, primarily $\alpha_v\beta_3$ and $\alpha_v\beta_5$ vitronectin-binding integrins, although other members of the family may be involved (reviewed by Shenk, 1996; Davison *et al.*, 1997). This interaction occurs between the integrin and an arg-gly-asp (RGD) motif present on each molecule of polypeptide III of the penton base.

Figure 1.3. The adenovirus life cycle.



The penton-integrin interaction triggers a series of events which see adenovirus-receptor complexes diffuse into clathrin-coated pits, where they are internalised by receptor-mediated endocytosis. This is a highly efficient process, with half of the adsorbed virus being internalised within ten minutes (reviewed by Shenk, 1996; Nemerow and Stewart, 1999). The internalised virus then escapes into the cytosol, with disruption of the early endosome triggered by a drop in pH and possibly involving the penton base protein (reviewed by Shenk, 1996). Prior to escape, sequential disassembly of the virion begins by selective dissociation and proteolytic degradation of specific proteins. Fibre, penton base, IIIa and VIII are removed prior to movement of the virus into the cytosol. Once in the cytosol, polypeptide VI is degraded, possibly by the virus-coded protease. Polypeptide IX is also lost and the DNA-containing core is freed from the remaining hexon shell (Greber *et al.*, 1993; reviewed by Shenk, 1996). During the disassembly process the virus moves towards the nuclear envelope by a process involving microtubules, possibly mediated via an interaction with the hexon protein (Suomakinen *et al.*, 1999). Upon reaching the nucleus the viral genome enters via nuclear pores, the genome which is still associated with polypeptide VII then associates with the nuclear matrix via its terminal protein (Schaack *et al.*, 1990; Fredman and Engler, 1993; Greber *et al.*, 1997).

1.3.5.2. Early gene expression.

Transcription of early genes begins 1-2 hours after the virus comes into contact with the cell. Both transcription and replication of the viral genome occur within discrete regions of the nucleus, termed inclusion bodies, or replication centres (Pombo *et al.*, 1994), with transcription shifting to the periphery of these bodies as DNA replication proceeds. As a rule, early gene products are involved in preparing the host cell for adenovirus replication. They mediate viral gene expression and DNA synthesis, induce cell cycle progression, block apoptosis, and antagonise a variety of host cell antiviral measures, as discussed below.

1.3.5.2.1. The E1 region.

The products of the adenovirus E1A gene are the first polypeptides to be synthesised during viral infection. This gene produces five mRNAs through

differential splicing with the two most abundant and important being the 12S and 13S mRNAs, which encode 243- and 289-amino acid proteins, respectively (termed 243R and 289R). Both proteins have identical amino and carboxy-terminal ends; they differ from each other in that the 243R protein lacks an internal stretch of 46 amino acids, designated conserved region 3 (CR3). The E1A proteins are constructed of three of these highly conserved regions (CR1, CR2 and CR3) separated by less highly conserved domains. The major function of these proteins is in activation of viral and cellular genes, either through direct interaction with the basal transcription machinery, or through interaction with upstream acting transcription factors. The E1A-13S protein can activate transcription through the TATA motif, resulting from binding of the CR3 region directly to the TATA binding protein (TBP), which is the DNA binding subunit of cellular transcription factor IID (TFIID). One method of transcriptional activation involves the tumour suppressor protein p53, which is known to repress transcription by binding to TBP; E1A-13S binding to TBP displaces p53 and relieves p53-mediated repression (Horikoshi *et al.*, 1995). Both E1A-12S and E1A-13S are capable of activating transcription by interacting with upstream transcription factors, one such example being the cellular factor E2F. E2F forms a complex with the cellular retinoblastoma tumour suppressor protein (pRb) which serves to repress transcription. E1A proteins can bind to pRb, mediated by the CR1 and CR2 domains and so displace E2F. This release of active E2F causes expression of cellular genes necessary to allow the cell to progress into S phase, which is the optimal environment conducive to viral replication. In addition E1A proteins can bind to the cellular transcriptional activator p300, this renders p300 incapable of binding to the transcription initiation complex and transcription is repressed. Genes regulated by p300 might encode proteins involved in cell cycle regulation. Therefore E1A inactivation of p300 could deregulate the cell cycle and induce cells to enter S phase (reviewed by Akusjarvi, 1993; Jones, 1995; Nevins, 1995; Shenk, 1996).

The deregulation of cell cycle control by E1A results in the accumulation of high levels of p53 in the nucleus. Normally p53 accumulation is associated with either growth arrest or apoptosis, in the presence of E1A apoptosis results. The E1B gene encodes proteins whose functions allow adenovirus to replicate successfully through blocking E1A-induced apoptosis (reviewed by Chinnadurai, 1998; White, 1998). Inhibition of apoptosis is largely achieved by the E1B-19K protein, which is a homologue of the cellular antiapoptotic protein Bcl-2. E1B-19K acts downstream in

the apoptosis pathway by an as yet unidentified mechanism, and is capable of blocking p53-mediated apoptosis and also apoptosis triggered by tumour necrosis factor (TNF)- α and anti-Fas antibodies. The E1B-55K protein also plays a role in inhibition of apoptosis by binding directly to the amino terminus of p53, which inhibits p53 function. Another important function of E1B-55K protein is its involvement in the differential control of viral gene expression, which it affects in conjunction with the E4-Orf6 protein, as will be discussed in section 1.3.5.2.4.

1.3.5.2.2. The E2 Region.

The E2 transcription unit encodes three proteins, all of which are vital for viral DNA replication. These are the 72-kDa single stranded (ss) DNA binding protein (DBP, encoded by E2A), the 80-kDa precursor terminal protein (pTP, encoded by E2B) and the 140-kDa DNA polymerase (Adpol, encoded by E2B). The exact roles of these proteins in viral DNA replication will be discussed in section 1.3.5.3. Besides its role in DNA replication, DBP is also involved in repression of E2 transcription, mRNA stability, virus assembly and host range function.

Activation of the E2 promoter is regulated by the E1A-13S protein. In the absence of E1A expression, constitutive expression from the E2 promoter is low. Upon expression of E1A, transcription from the E2 promoter increases, mediated through the dissociation of E2F and pRb (see section 1.3.5.2.1), allowing E2F to bind to and activate the E2 promoter (reviewed by Swaminathan and Thimmapaya, 1996).

1.3.5.2.3. The E3 Region.

The E3 region of Ad5 gives rise to nine overlapping mRNAs that are alternatively processed from a common pre-mRNA that initiates from the E3 promoter. Of these nine predicted proteins, six have been identified in infected cells. These include the gp19K, 10.4K, 14.5K and 14.7K proteins, so called because of their molecular weights, which are involved in helping the virus evade the host immune responses directed against it. The E3 region is only required for viral replication *in vivo* and can be deleted without affecting growth in cell culture (reviewed by Wold and Gooding, 1991; Wold and Tollefson, 1998).

The E3-gp19K protein binds to and retains newly synthesised major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum. This class of MHC molecule is involved in presenting endogenous peptides to CD8⁺ cytotoxic T-lymphocytes (CTLs), normally resulting in lysis of the presenting cell. Thus gp19K expression results in down-regulation of CTL-mediated lysis of adenovirus infected cells (reviewed by Blair and Hall, 1998).

E3-14.7K protein inhibits TNF-induced apoptosis, possibly by interfering with the signalling pathway by which TNF exerts its effects after binding to the cellular receptor TNFR1 (reviewed by Wold and Tollefson, 1998). Recently, a cellular protein named FIP-3, a member of the GTPase superfamily of signal transducers, was discovered due to its interaction with E3-14.7K. This raises the possibility that FIP-3 is the protein through which 14.7K mediates its anti-apoptotic effects (Li *et al.*, 1999).

The E3-10.4K and 14.5K proteins associate to form a complex termed RID, an acronym for receptor internalisation and degradation. Both 10.4K and 14.5K are integral membrane proteins and localise to the plasma membrane and intracellular vesicles (reviewed by Wold and Tollefson, 1998). RID prevents TNF-induced apoptosis by inhibiting translocation of cytosolic phospholipase A₂ (cPLA₂) to membranes (Dimitrov *et al.*, 1997). Cleavage of arachidonic acid from membrane glycolipids, mediated by cPLA₂, is an important signal in TNF-induced apoptosis. This action of RID could explain how it blocks TNF-induced apoptosis and contributes to the survival of the infected cell. In addition, the RID complex also stimulates internalisation of the cell surface receptor Fas (Shisler *et al.*, 1997). These receptors function by inducing apoptosis when engaged by Fas ligand (FasL) expressed on the surface of CTLs. Thus RID expression is another way in which adenovirus can down-regulate CTL-mediated cell lysis.

In addition to proteins involved in regulation of the host immune response, the E3 region also encodes an 11.6 kDa membrane glycoprotein, termed the adenovirus death protein (ADP). ADP ultimately localises to the nuclear membrane and facilitates the release of progeny virions by triggering cell lysis. The mechanism by which ADP achieves this is poorly understood (Tollefson *et al.*, 1996a, 1996b).

1.3.5.2.4. The E4 Region.

The E4 region encodes one or more functions that are required for lytic growth in cell culture. These include DNA replication, mRNA stability and host cell shutoff (reviewed by Leppard, 1997). Primary transcripts from the E4 promoter are alternatively spliced to produce at least 18 distinct mRNAs. These are predicted to encode for seven polypeptides termed Orf1 – Orf4, Orf6, Orf3/4 and Orf6/7. All these have been demonstrated to exist in infected cells with the exception of the Orf3/4 protein.

The Orf6 protein functions as a complex with the E1B-55K protein and facilitates the export of viral mRNAs from the nucleus and also inhibits the export of most cellular mRNAs (reviewed by Imperiale *et al.*, 1995). It can also bind to p53 and block p53-induced apoptosis (Dobner *et al.*, 1996), a process that is independent of the presence of E1B-55K, which separately plays a role in disrupting p53 function. The Orf3 protein can initiate the reorganisation of specialised structures in the nucleus known as promyelocyte oncogenic domains (PODS; Carvalho *et al.*, 1995, Doucas *et al.*, 1996), although the reason for this in the context of viral infection is unclear. Additionally, both Orf3 and Orf6 can independently improve the stability of late RNA in the nucleus (Ohman *et al.*, 1993; Nordqvist *et al.*, 1994) and they play an as yet undefined role in regulation of Ad DNA replication (reviewed by Leppard, 1997). The activities of these two proteins are apparently redundant in lytic infection, either one is sufficient for efficient virus production (Bridge and Ketner, 1989; Huang and Hearing, 1989a).

Of the remaining proteins, Orf6/7 regulates the Ad E2 promoter, an effect mediated through its ability to bind to and stabilise E2F dimers bound at the promoter (Huang and Hearing, 1989b). The Orf4 protein is important in down-regulating the expression of E1A and E4 genes, an effect mediated through its interaction with protein phosphatase 2A (PP2A; Bondesson *et al.*, 1996). Orf2 is a soluble cytoplasmic protein with no known function (Dix and Leppard, 1995) and Orf3/4 has yet to be detected in infected cells but is predicted to exist based on Ad2 mRNA analysis (Virtanen *et al.*, 1984).

1.3.5.2.5. The delayed early genes.

The genes encoding polypeptides IX and IVa2 are designated delayed early due to the time in the infectious cycle at which they are expressed, around the time of the onset of viral DNA replication. They are not regarded as true late genes as their expression does not absolutely depend on viral DNA replication. IX has been reported to be incorporated into the mature viral capsid, where it strengthens hexon-hexon interactions (Boulanger *et al.*, 1979). pIX has also been shown to act as a transcriptional activator of TATA-containing promoters (Lutz *et al.*, 1997). The IVa2 protein may have a role in viral DNA encapsidation (Zhang and Imperiale, 2000) in addition to its function as a transcriptional activator of the major late promoter (MLP), which is discussed in detail in section 3.4.

1.3.5.3. DNA replication.

DNA replication begins about 5 hours after infection in HeLa cells and continues until the host cell dies (reviewed by Shenk, 1996). The E2 gene encodes the three viral replication proteins (section 1.3.5.2.2.) which are capable of initiating DNA replication, although this is inefficient and chain elongation is limited. Efficient DNA replication requires three additional cellular proteins, nuclear factors (NF) I, II and III (reviewed by De Jong and Van der Vliet, 1999). The sequences needed in *cis* for replication are located within the terminal 51 bp at either end of the genome. Within this origin of replication (*ori*) are three regions, an essential core region and two auxiliary regions that enhance the efficiency of DNA replication. The auxiliary regions bind NFI and NFII, which enhances recruitment of pTP-Adpol by the core region. Binding of NFI seems to be enhanced by the presence of DBP which forms a regular multimeric protein-DNA complex with the ds DNA (Stuiver and Van der Vliet, 1990).

Replication of the genome begins with the preterminal protein (pTP) acting as a primer to initiate replication exactly at the genome termini, so preserving the integrity of the ITRs during multiple rounds of DNA replication. An ester bond is formed between the β -OH group of a serine residue in pTP and the α -phosphoryl group of dCMP, the first residue at the 5' end of the DNA chain. This reaction requires the presence of the viral polymerase (Adpol) and occurs after the pTP-Adpol complex is

correctly positioned on the template. The resulting 3'-OH group of the pTP-dCMP complex then serves to prime DNA synthesis (reviewed by Shenk, 1996).

Chain elongation requires the other E2 proteins, Adpol and DBP, and a cellular protein NFII. DBP facilitates chain elongation by unwinding the dsDNA molecule and is displaced by Adpol as the nascent chain of DNA is synthesised. NFII has topoisomerase activity and is required for chain elongation to proceed beyond 9 kbp of the genome (Nagata *et al.*, 1982). The ssDNA chain produced is stabilised by binding of DBP, which protects it from nucleases and self-hybridisation.

Synthesis of a complete genome occurs by one of two mechanisms; termed Type I and Type II replication. In Type I replication Adpol synthesises DNA from one terminus of the linear DNA molecule to the other, resulting in the production of a new viral genome with a displaced single strand. In Type II replication the complementary ends of the displaced strand anneal to form a panhandle structure, with the duplex region forming a complete ITR element. This ITR allows DNA replication to proceed in the same manner as for the ds genome (reviewed by Van der Vliet, 1995).

1.3.5.4. Late gene expression.

Late genes begin to be expressed efficiently at the onset of viral DNA replication. All late proteins are encoded by the major late transcription unit (MLTU) which is under the control of the major late promoter (MLP). The MLP is active at basal levels during the early phase, with its activity increasing to high levels after the onset of viral DNA replication (Shaw and Ziff, 1980). The MLTU encodes the structural proteins necessary for the construction of the viral capsid and also the scaffold proteins necessary for its correct assembly. The regulation of the MLP is of particular relevance to this thesis and is discussed in detail in section 3.4.

1.3.5.4.1. Splicing and polyadenylation of late pre-mRNA.

The adenovirus MLTU generates a primary transcript of approximately 28,000 nucleotides that can be processed into a minimum of 20 cytoplasmic mRNAs (figure 1.4). These mRNAs are grouped into five families (L1 to L5), each consisting of multiple species which have their poly (A) tails in the same position. All MLTU mRNAs have a common 5' leader sequence, the so-called tripartite leader, which is

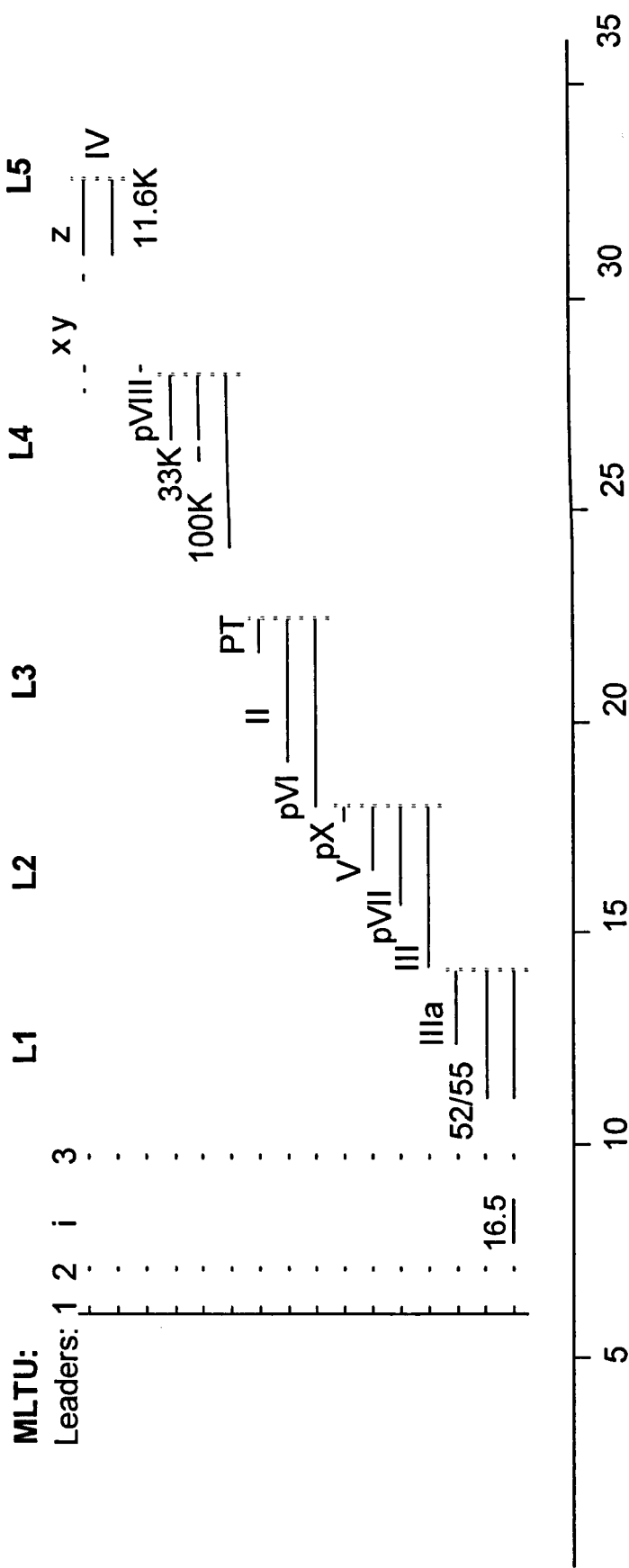


Figure 1.4. Human adenovirus type 5 major late transcription unit (MLTU).

The virus genome is represented in the centre by a line scale, with numbering indicating the distance from the conventional left end in kb. Late gene regions are shown in boldface. mRNAs are shown as solid lines, with gaps to indicate introns; the major late promoter is indicated by a solid vertical line, with polyadenylation sites designated by broken vertical lines. Proteins translated from mRNAs are indicated adjacent to the mRNA.

spliced to the body of the mRNA sequence. Therefore, the MLTU is an example of an alternatively spliced gene in which one 5' splice site (leader 3) can be spliced to more than 15 different 3' splice sites. The 5' tripartite leader (TPL) is a 201 nt sequence consisting of three short exons 1,2 and 3 (Berget *et al.*, 1977; Chow *et al.*, 1977). An additional exon, the 440 nt i leader, may be included between exons 2 and 3. Inclusion of the i leader is temporally regulated by two Ad proteins, E4-Orf3 and E4-Orf6 (Ohman, 1993; Nordqvist *et al.*, 1994), such that it is included in mRNAs produced from the MLTU at early times, but is usually excluded at late times. The function of the i leader sequence and the 16.5 kDa protein it encodes are unclear (Symington *et al.*, 1996). The role of the TPL sequence in translation of late mRNAs is discussed in section 1.3.5.4.2.

Transcription of the MLTU occurs during both early and late phases. The transcription start site is the same both early and late (Shaw and Ziff, 1980) but the length of the primary transcript and the structure of the resulting mRNAs differ considerably (Chow *et al.*, 1979). At early times, transcription from the MLP is subjected to a control at the level of transcription termination. At this stage the transcriptional activity decreases gradually over a large region beginning around map unit 40, with no polymerases extending beyond map unit 75 (Iwamoto *et al.*, 1986). At late times, the transcription termination block is alleviated and transcripts initiated at the same promoter continue to the right hand end of the genome. During the early phase of infection, only the L1 poly (A) site is utilised, despite the fact that transcription proceeds across the L2 and L3 poly (A) sites (Akusjarvi and Persson, 1981; Nevins and Wilson, 1981; Iwamoto *et al.*, 1986). The presence of a *cis*-acting selector element upstream from the L1 poly (A) site is thought to be responsible for the preferential L1 production at this stage (De Zazzo and Imperiale, 1989). Late after infection the situation is reversed and the L2 and L3 poly (A) sites are used more efficiently than the L1 site (Nevins and Wilson, 1981).

Alternative splicing within the L1 gene family has been widely studied and involves a choice between two alternative 3' splice sites. The L1 region encodes two major mRNAs, the so-called 52/55K (proximal 3' splice site) and IIIa (distal 3' splice site) mRNAs. 52/55K mRNA is the only mRNA expressed at early times of infection, while IIIa mRNA splicing is confined to the late phase (Shaw and Ziff, 1980; Akusjarvi and Persson, 1981; Nevins and Wilson, 1981). Studies have shown that the shift from only 52/55K to both 52/55K and IIIa mRNA splicing results from a

reduction in 52/55K 3' splice site usage, combined with an enhanced recognition of the IIIa 3' splice site (Kreivi and Akusjarvi, 1994).

1.3.5.4.2. Nuclear export and translation of late mRNAs.

During the late phase of infection, cellular protein synthesis is shut off, due to a translational block of host cell mRNAs (reviewed by Zhang and Schneider, 1993). In addition, most cellular mRNAs fail to accumulate in the cytoplasm despite continued nuclear synthesis and processing (Beltz and Flint, 1979). In contrast, late viral mRNAs are selectively exported to the cytoplasm and are efficiently translated late after infection (Babich *et al.*, 1983; Binger and Flint, 1984).

The selective accumulation of viral mRNAs during the late phase of infection is mediated by the Ad encoded E1B-55K and E4-Orf6 proteins, which form a specific complex within infected and transiently transfected cells (Sarnow *et al.*, 1984; Goodrum *et al.*, 1996). The E1B-E4 protein complex appears to modulate viral and cellular mRNA transport after transcription and processing but before translocation of mRNAs through the nuclear pores (Leppard and Shenk, 1989). Studies have shown that both proteins are localised within and about the periphery of viral nuclear inclusion bodies (Ornelles and Shenk, 1991) which are believed to be the sites of viral replication (Puvion-Dutilleul and Puvion, 1991). This observation is consistent with the idea that the E1B-E4 complex regulates RNA metabolism at an intranuclear step, possibly by facilitating the movement of mature viral mRNA to the nuclear pore complex (Dix and Leppard, 1993; Leppard, 1993; Nordqvist *et al.*, 1994). The mechanism by which the E1B-E4 complex regulates mRNA transport may be more complicated than originally envisioned. The complex has been shown to continuously shuttle between the nucleus and cytoplasm (Dobbelstein *et al.*, 1997), suggesting an alternative theory for how it regulates mRNA export.

In addition to their facilitated transport from the nucleus, viral mRNAs are preferentially translated when they reach the cytoplasm. Three virus-encoded functions, VA1 RNA, the tripartite leader sequence (TPL) and the L4-100K protein have been shown to increase translational efficiency during the late phase of infection.

Following infection by a number of viruses, EIF-2 α kinase inhibits translation by phosphorylating the α subunit of the initiation factor EIF-2 (Reichel *et al.*, 1985). It has been proposed that VA1 RNA functions by binding to EIF-2 α kinase, thus

blocking its activation and preserving the function of EIF-2 (Katze *et al.*, 1987). VA1 RNA has been shown to co-purify with viral mRNAs (Mathews, 1980) and, as a result, they might protect viral but not cellular protein synthesis, providing a functional compartmentalisation.

The TPL sequence increases translation of heterologous mRNAs during the late, but not the early phase of infection and does not increase translational efficiency when placed at the 3' end of mRNA, suggesting a role in initiation (Logan and Shenk, 1986). The TPL sequence lacks secondary structure and it has been proposed that TPL-containing viral mRNAs can continue to be translated late after infection because the 40S ribosome can scan from the cap to AUG codon without the need for a helicase as eIF-4F activity becomes limiting due to inactivation late during adenovirus infection (Huang and Schneider, 1991). In contrast, most cellular mRNAs are no longer translated in the absence of eIF-4F because they require the helicase to permit scanning through the more extensive secondary structure at their 5' ends.

Finally, there is a selective activation of viral late protein synthesis by the L4-100K protein (Hayes *et al.*, 1990) by an as yet unknown mechanism. The L4-100K protein has been shown to bind to mRNA (Adam and Dreyfus, 1987; Riley and Flint, 1993), suggesting it may function at the polysome to facilitate viral translation.

1.3.5.5. Virus assembly and release.

Structural proteins along with scaffolding proteins are transported to the nucleus, the site of virus assembly (reviewed by D'Halluin, 1995; Shenk, 1996). Capsids are formed through a number of stages; firstly trimeric hexon capsomeres are rapidly assembled, a process which requires the scaffolding protein L4-100K. Penton capsomeres, consisting of the penton base and trimeric fibre protein, assemble more slowly. After their production, hexon and penton capsomeres accumulate in the nucleus where they associate to form light intermediates. Into these are inserted the viral DNA; the *cis*-acting sequences necessary for encapsidation of the genome reside between bp 194 and 358 in Ad5 (reviewed by Schmid and Hearing, 1995). Cellular factors that bind to these sequences may play a role in DNA encapsidation (Schmid and Hearing, 1998). Also the L1 52/55K protein, in addition to its role as a scaffolding protein (Hasson *et al.*, 1992), may also be involved in DNA encapsidation (Gustin and Imperiale, 1998). Packaging of the genome leads to the formation of

heavy intermediates, which are converted to mature virions through the proteolytic cleavage of the precursor forms of several virion polypeptides such as pTP, pVI, pVII and pVIII. These cleavage events are mediated by the encapsidated viral protease and function to stabilise the structure and render it infectious.

Release of the mature virions from the infected cell is facilitated by disruption of the cytoskeleton in response to the E1B-19K protein and the viral protease. These proteins weaken the integrity of the cell and leave it susceptible to lysis by mechanical stress (Chen *et al.*, 1993). An additional protein required for efficient cell lysis is the adenovirus death protein (ADP), an 11.6kDa protein encoded by the E3 region (Tollefson *et al.*, 1996a, 1996b).

1.4. Adenovirus vectors.

Adenoviral vectors represent an important class of gene delivery vehicle for *in vivo* gene therapy. There are several major advantages of adenoviral vectors that make them ideally suited to gene therapy applications. Their structure, genome organisation and replication cycle are all well characterised, as are the functions of individual proteins and their interactions with cellular factors. The safety profile of adenovirus is well established; live, encapsulated strains of serotypes 4 and 7 have been used as an oral vaccine to vaccinate military personnel against acute respiratory infections for a number of years (Top *et al.*, 1971). Also, the serotypes used most commonly as gene therapy vectors (2 and 5) do not generally cause serious disease, being associated with mild self-limiting infections of the upper respiratory tract. Adenoviral vectors can be grown to high titres (in excess of 10^{11} plaque forming units (pfu) per ml) and easily purified making them suitable for *in vivo* applications. In contrast to retroviruses that can only infect actively dividing cells, adenoviruses can infect and deliver their DNA directly to the nucleus, without the need for cell division, enabling them to transduce both dividing and non-dividing cell types. Additionally, they are able to infect a wide variety of cell types due to the ubiquitous nature of the CAR receptor.

1.4.1. Construction of adenovirus vectors.

Gene delivery using adenovirus faces two initial problems, a wild type virus can only efficiently package approximately 2 kbp of exogenous DNA (Ghosh-Choudhury *et al.*, 1987) and such vectors would be replication-competent *in vivo*, leading to infection and spread of the virus. To address these problems the viral genome is engineered to remove essential gene functions and create extra capacity for insertion of heterologous sequences. The E3 region is non-essential for growth in tissue culture and can be deleted without having any adverse effects on virus growth *in vitro*. The E1 region is essential for viral growth, and represents an ideal region for deletion in order to render the virus replication-defective. To allow the virus to grow in tissue culture (essential for production of vector stocks) the missing E1 functions must be provided *in trans*. The existence of a human embryonic kidney cell line (293 cells; Graham *et al.*, 1977) has allowed the isolation and propagation of a number of viruses with mutations and deletions in the E1 region (reviewed by Shenk and Williams, 1984). A combination of deletions in the E1 and E3 regions allows the insertion of

approximately 8 kbp of foreign DNA. Vectors engineered in this way were the first to be developed and used in pre-clinical and clinical trials, and are discussed in section 1.4.2.

A scheme for constructing a recombinant adenovirus is depicted in figure 1.5. Because of the size of the genome (and the resulting high frequency of restriction enzyme sites), it is difficult to directly clone any heterologous DNA into the genome. Instead, homologous recombination is used. The heterologous sequence is cloned into a plasmid that contains the left end of the viral genome, such that the inserted gene is flanked on one side by the ITR (0 – 1 map units) and on the other by approximately 2 kb of Ad sequence (9 – 16 map units), so that it replaces the E1 region. This plasmid is linearised and transfected into 293 cells, along with linear viral DNA backbone that has been digested to remove the left end. Homologous recombination then occurs between the plasmid and viral DNA and recombinant virus that contains the inserted transgene is produced. The recombinant virus is only infectious in 293 cells, which provide E1 functions *in trans*. For reviews of the construction and use of adenovirus vectors for gene therapy, see Kozarsky and Wilson, 1993; Trapnell and Gorziglia, 1994; Bramson *et al.*, 1995 and Benihoud *et al.*, 1999.

1.4.2. First generation adenovirus vectors.

The so-called first generation vectors, those deleted for E1 and E3, are the largest group of adenovirus vectors so far studied for the purpose of gene therapy. The range of applications for which they have been investigated is beyond the scope of this section (reviewed by Kozarsky and Wilson, 1993; Trapnell and Gorziglia, 1994), instead this section will concentrate on those studies which first revealed the limitations of these vectors and prompted their further development.

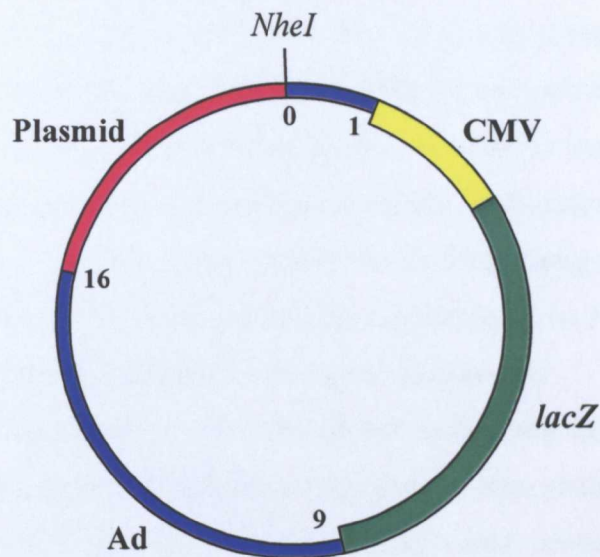
Due to their natural tropism for the respiratory tract, adenoviruses have been investigated as candidates for gene therapy of the two most common inherited gene defects which generate pathology in the lung, cystic fibrosis (CF) and α_1 -antitrypsin (α_1 -AT) deficiency. Studies by Rosenfeld *et al.* (1991) showed that α_1 -AT delivered by tracheal instillation to cotton rats using an E1/E3-deleted Ad5 vector, could be expressed and detected in epithelial lining fluid for at least one week. Further studies by the same group (Rosenfeld *et al.*, 1992) showed that the chloride ion secretory defect of CF could be corrected in an *in vitro* model and was also expressed in cotton

Figure 1.5. Generation of recombinant adenoviruses by homologous recombination.

- (a) A plasmid containing the left-most 15 map units (5800 bp) of Ad DNA, with a transgene (e.g. CMV-driven β -galactosidase) replacing Ad E1 sequences, is constructed. The plasmid sequences indicated (red) contain the ampicillin resistance gene and bacterial origin of replication.
- (b) Plasmid is linearised (with *NheI* in this example) and transfected into 293 cells along with *ClaI*-digested non-infectious viral DNA lacking the left end.
- (c) Recombination occurs between the homologous Ad sequences (9 – 16 map units), and recombinant adenovirus containing the *lacZ* reporter gene but deleted for E1 is produced.

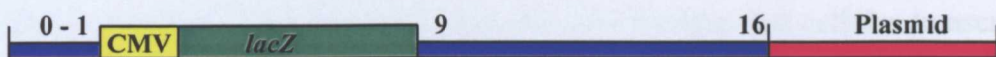
Numbers indicate map units of the Ad genome and the diagonal lines represent a length of DNA not shown.

(a).



(b).

Linearised plasmid.



*Cla*I-digested Ad DNA



Co-transfection into 293 cells
followed by homologous
recombination.

(c).



rats. They used an E1/E3-deleted Ad5 vector to deliver the cDNA encoding the normal human cystic fibrosis transmembrane conductance regulator (CFTR) to the lungs of cotton rats. CFTR RNA transcripts could be detected for up to 6 weeks, with protein detected up to 14 days post-infection. In another report Englehardt *et al.* (1993b), using a human bronchial xenograft model in nude mice, demonstrated that an E1-deleted Ad5 vector could transduce 5-20 % of human lung surface epithelial cells with the human CFTR. This expression was detectable up to 5 weeks post-infection, with the protein correctly localised to the apical membrane.

The study of Englehardt *et al.* (1993b) was among the first to address what has become one of the major disadvantages of first generation vectors; residual viral gene expression. It revealed that the E1-deleted vector used, when administered at high multiplicity of infection, expressed high levels of the E2A-encoded early protein DBP in a small subset of cells. However, no expression of the late proteins hexon or fibre and no replication-competent virus was detected. These results suggested that the vector was prevented from entering the late phase of the replication cycle in the absence of E1A and E1B, but the E2A promoter was activated in a subset of cells, possibly via the action of E1-like cellular proteins, or pre-existing E1 sequences. This hypothesis is supported by the previously reported finding that cellular transcription factors, such as NF-IL6, can compensate for a lack of E1A (Spergel *et al.*, 1992). Indeed, E1-deleted virus has long been known to be replication-competent at high multiplicities of infection (Nevins, 1981).

The first clinical trial for gene therapy of CF was that of Crystal *et al.* (1994). Using the vector of Rosenfeld *et al.* (1992) they showed that the normal CFTR cDNA could be expressed in the respiratory epithelium, with no adverse effects evident at doses up to 2×10^7 pfu. However, a transient systemic and pulmonary syndrome was observed in one patient who received a dose of 2×10^9 pfu. This was most likely caused by vector-induced inflammation of the lower respiratory tract, a hypothesis supported by the patient having high serum levels of interleukin (IL) 6. Despite this, no virus was shed, ruling out the possibility that IL-6 had induced vector replication by inducing the E1A-like transcription factor NF-IL6. Although the trial showed CFTR expression, it failed to show the correction of any of the clinical defects of CF.

Another trial by Zabner *et al.* (1993) showed that the chloride ion transport defect in patients with CF could be corrected in nasal epithelium using CFTR cDNA delivered by an E1-deleted Ad2 vector. However, the correction was only evident for

three weeks post-therapy and as with the study by Crystal *et al.* (1994), significant inflammatory reactions were observed in patients receiving the highest vector doses. Subsequent studies by Knowles *et al.* (1995) failed to show any correction of the CF defect using an E1/E3 deleted Ad5 vector, although this could be due to differences in their protocol, including the use of a different vector construct and an alternative method of measuring the potential difference across the nasal epithelium.

In addition to CF gene therapy, first generation vectors were also being used in other applications. The feasibility of intramuscular injection of adenovirus vectors was demonstrated by several groups (Quantin *et al.*, 1992, Stratford-Perricaudet *et al.*, 1992, Ragot *et al.*, 1993). The study by Ragot *et al.* (1993) showed that the minidystrophin cDNA could be delivered and expressed in muscle fibres of dystrophin-deficient *mdx* mice, raising the possibility for gene therapy of Duchenne muscular dystrophy. Expression of the minidystrophin cDNA could be detected in 5 – 50 % of the injected muscle fibres; this expression declined to 20 % over 13 weeks.

Other studies using first generation vectors reported similar results; efficient gene transfer accompanied by an immune response and transient transgene expression (reviewed by Kozarsky and Wilson, 1993; Trapnell and Gorziglia, 1994; Bramson *et al.*, 1995). Despite the mixed results of these first trials, they did show the limitations of the vectors used, mainly the fact that they were capable of expressing viral genes and were not completely replication-deficient. The immune response to the vectors was also a potential problem, ruling out the possibility of using repeat doses to compensate for the transient transgene expression (Yei *et al.*, 1994). In addition to this, vector stocks could be contaminated by replication-competent virus produced by homologous recombination between the vector and E1 sequence present in 293 cells.

The most tragic and unexpected outcome of a clinical trial occurred in 1999 at the University of Pennsylvania's Institute of Human Gene Therapy (IHGT). Jesse Gelsinger, an 18-year-old volunteer in a Phase I clinical trial, died on September 17, 1999 after complications arose following treatment with an adenovirus vector. Gelsinger was one of 18 participants in the trial to develop a treatment of ornithine transcarbamylase (OTC) deficiency, which is an inherited enzyme deficiency resulting in a block to ammonia metabolism in the liver. Gelsinger received 3.8×10^{13} particles, the highest dose of vector in the trial, delivered directly into the hepatic artery. However, post-mortem examination detected vector and OTC transgene sequences in other organs in addition to those in the target organ, the liver. The

autopsy also revealed that the major factor in Gelsinger's death was oxygen deprivation brought on by Adult Respiratory Distress Syndrome (ARDS), a severe lung dysfunction. Immediately following vector administration there was an innate immune response, followed by injury to the liver and inappropriate blood coagulation. His underlying genetic defect made it difficult for him to handle the stress of the immune activation, resulting in an accumulation of ammonia in the blood, and then coma. The onset of ARDS occurred three days later, which could not be reversed in the time leading to his death. It was also discovered that complications might have arisen due to Gelsinger's bone marrow being depleted of erythroid precursor cells, suggesting an undetected genetic condition or a pre-existing parvovirus infection. Following this event the Food and Drug Administration (FDA) stopped all eight trials run by the IHGT after finding "serious deficiencies" in the monitoring of clinical trials. A full report can be found on the University of Pennsylvania's Institute of Human Gene Therapy homepage (<http://www.med.upenn.edu/ihgt/jesse.html>). The results of this trial highlight that it is not only vector design that has to be improved, but also the quality control and monitoring of clinical trials.

1.4.3. The immune response to adenovirus vectors.

After administration, first generation vectors elicit an immune response which is both cellular and humoral in nature. This response is similar to that seen in a *wt* virus infection (Prince *et al.*, 1993), with an initial influx of inflammatory cells and the release of TNF α and interleukins (IL-1 β , IL-6 and IL-8). This initial inflammatory response does not appear to require expression of adenovirus or transgene encoded proteins and may even be triggered by the infection process itself (Bruder and Kovesdi, 1997). Immunoclearance of infected cells then occurs, mediated by MHC class I-restricted CTLs directed towards viral antigens and/or the transgene product. During Ad5 infection, the major epitopes for CTLs are generated by the products of the E1A region (Routes *et al.*, 1991). Thus the strength of the immune response against E1A-deleted vectors was unexpected. In order to improve the effectiveness of adenovirus vectors, studies were initiated to define the nature of the immune response responsible for the elimination of transgene expression and development of pathology in transfected tissues.

Studies by Yang *et al.* (1994a) investigated adenovirus-mediated gene transfer to the livers of immunocompetent (CBA) and genetically athymic (*nu/nu*) mice. An E1/E3-deleted vector, expressing a *lacZ* reporter gene, was infused into the biliary tract of female mice and longevity of transgene expression monitored. Over the duration of the study (60 days), no reduction in expression of *lacZ* was observed in *nu/nu* mice, whereas in CBA mice *lacZ* expression was undetectable by day 21. Persistence of viral DNA in *nu/nu* but not in CBA mice was also apparent after Southern blot analysis. Analysis of splenocytes harvested from mice after 14 days showed that CBA mice had MHC-restricted CTL activity, indicating the involvement of these cells in the immune response against adenovirus vectors. The detection of low levels of E2A-DBP and hexon protein suggested that the cellular immune response was directed against *de novo* synthesised viral antigens.

Further studies in mice by Yang *et al.* (1995) determined that MHC class I-restricted CD8⁺ CTLs were activated in response to newly synthesised viral antigens, leading to destruction of transduced cells and loss of transgene expression. MHC class II-restricted CD4⁺ T helper cells, activated by exogenous viral antigens, were insufficient to completely eliminate transgene-containing cells in the absence of CD8⁺ T cells. However, they did contribute to the formation of neutralising antibodies in the airway that would block any subsequent adenovirus-mediated gene transfer. Subsequent work showed that a response by CD4⁺ cells alone was sufficient to destroy transduced hepatocytes, although the possibility of contaminating CD8⁺ cells was not excluded (Yang and Wilson, 1995). These results supported a then recent finding of Bendelac *et al.* (1994) that a subset of CD4⁺ T cells could be selected by MHC class I molecules and contribute to the clearance of viral infection.

Even though the nature of the immune response to Ad5 vectors was becoming clearer, it was still undetermined as to whether it was the expression of transgene or viral proteins that was responsible for the response. Studies by Yang *et al.* (1996b) showed that transfer of the *lacZ* gene to the lungs of mice transgenic for (and therefore tolerant of) *lacZ* resulted in a CTL response and loss of transgene expression. Similar results were found when using the gene for ornithine transcarbamylase (OTC), an endogenous protein found in the liver. These results indicated that CTLs specific for viral antigens were responsible for transgene instability and that CTLs specific for the transgene product alone could not account for the observed problems. In contrast to these findings, Tripathy *et al.* (1996) showed

definite involvement of an immune response directed at the transgene-encoded protein. In their study immunocompetent mice were injected intramuscularly with identical Ad vectors encoding either self (murine) or foreign (human) erythropoietin (AdmEpo and AdhEpo respectively). Mice injected with AdmEpo had an increase in haematocrit when compared to mice that received a control virus lacking a transgene. In contrast, mice injected with AdhEpo showed significantly lower haematocrits than the control, indicating that expression of hEpo had reduced the number of red blood cells. When the AdhEpo virus was administered to CD8⁺ T cell-deficient mice, haematocrit levels were similar to those seen in immunocompetent mice receiving AdmEpo. These results suggested that the CD8⁺ T cell response directed against transduced cells was directed at the transgene-expressed protein. Studies by Wadsworth *et al.* (1997) also showed that there was a T cell response against foreign transgenes, but it was ineffective at eliminating transduced cells. To add further confusion, studies by Sparer *et al.* (1997) and Jooss *et al.* (1998a) showed that the recognition of Ad epitopes by CTLs was dependent on the MHC haplotype of the mouse strain used. In C57BL/6 mice the viral hexon protein contained the immunodominant epitope, whereas in C3H mice it was the alkaline phosphatase transgene protein. As the human population has diverse HLA haplotypes, this would present another problem to be overcome when designing vectors for human gene transfer applications.

In summary, administration of first generation vectors elicits a bi-phasic immune response. Firstly an inflammatory response results in clearance of the majority of the vector. This is followed by a CTL response (mostly CD8⁺ but also CD4⁺) against viral or transgene encoded proteins, which leads to the destruction of the transduced cells. The epitope(s) recognised by the CTL are dependent on transgene origin, viral attenuation and the recipient's haplotype.

1.4.4. Second generation adenovirus vectors.

The limitations of first generation vectors prompted a major re-evaluation of adenovirus vector design. The problems of leaky viral DNA replication and accumulation of late gene products had to be addressed. To prevent these events it was necessary to further engineer the viral genome to remove essential gene functions. Adenovirus vector development has focused on crippling the virus by

deleting essential genes and complementing the missing function(s) *in trans* using engineered cell lines or viral helper systems. This multi-deficient vector approach has several advantages: increased capacity for transgene DNA; decreased incidence of replication-competent virus in vector stocks by requiring two or more independent recombination events to occur to generate such virus; addition of extra blocks to viral replication and protein expression, leading to a lower expressed viral antigen burden.

1.4.4.1. Modifications to the E2 region.

Adenovirus DNA replication, required for expression of late gene products, is regulated by many viral proteins encoded by the E1, E2 and E4 regions (reviewed by Shenk, 1996). Early attempts to improve first generation vectors focused on the introduction of mutations into the E2A-encoded DBP. Engelhardt *et al.* (1994) used the Ad5 temperature sensitive (*ts*) mutant H5*ts*125 (Klessig and Grodzicker, 1979) to generate a second generation vector. This E1/E3 deleted vector contains a *ts* mutation in the DBP that allows the virus to grow at the permissive temperature of 32 °C, but not at 39 °C. Studies using this vector *in vivo* showed prolonged expression of a *lacZ* reporter gene in mouse liver compared to that of a first generation vector (70 days, as opposed to 14 days). It was also observed that the immune response against the two vectors differed. The initial innate phase was the same against the two vectors, but in the second phase a marked reduction in infiltration of CD8⁺ T cells, together with a delayed and blunted inflammatory response was observed, adding weight to the previous suggestion that earlier vectors were not completely-replication deficient. Another study by Yang *et al.* (1994b) using the same vector, but this time administered to mouse lung, again showed improved persistence of *lacZ* expression and a reduced inflammatory response. Additional experiments using a similar vector containing CFTR also showed increased persistence when compared to an E1-deleted vector containing the same transgene. This increased persistence was attributed to the E2A *ts* mutation although it could have been due to the difference in E3 status as the second generation vector retained the majority of the E3 region. Pre-clinical trials in baboons gave similar results (Goldman *et al.*, 1995), although transgene expression from the E2A *ts* vector was not as prolonged as expression from an E1-deleted vector in immunodeficient mice. These results suggested that an immune response against second generation vectors was still responsible for clearance of transduced cells, an

effect Goldman *et al.* (1995) attributed to leaky expression of the E2A *ts* mutant at the intermediate temperature of 37 °C. This observation tied in with the results of the *in vitro* experiments of Engelhardt *et al.* (1994) that showed low but detectable levels of viral growth at 37 °C. These results suggested that a more complete block to viral DNA replication and late gene expression, possibly by deletion of the E2A gene, would be more effective.

In order to construct a vector containing a deletion in the E2A gene a complementing cell line was needed to provide the missing gene function *in trans*. Several publications describe the construction of cell lines expressing DBP (Brough *et al.*, 1992; Gorziglia *et al.*, 1996; Zhou *et al.*, 1996). The cell lines of Gorziglia *et al.* (1996) and Zhou *et al.* (1996) were used to grow vectors with partial deletions in the E2A gene, increasing the capacity for insertion of exogenous DNA to about 9 kbp. Evaluation of these vectors suggested that a partial deletion of E2A had no effect on the efficiency of transduction, while the persistence of transgene expression has yet to be evaluated *in vivo*. One potential problem is that E2A-deleted vectors are hindered by a 10-30 fold reduction in growth *in vitro*, even in complementing cell lines. Reduced viral replication and late gene expression is accomplished with these vectors; however, it is possible that deleting E2A also triggers enhanced expression from other viral genes such as E4 (Rice and Klessig, 1985). This could lead to other problems unless the E4 region is also deleted, an approach that is discussed in section 1.4.4.2..

Complementing cell lines expressing other E2 proteins have been described (Schaack *et al.*, 1995; Langer and Schaack, 1996; Amalfitano and Chamberlain, 1997; Amalfitano *et al.*, 1998). Moorhead *et al.* (1999) reported the construction of a vector with a deletion in the preterminal protein (pTP). This vector was shown to elicit a reduced immune response when injected intradermally into mouse ears. Duration of transgene expression was also increased when compared with a first generation vector. Results from this and the study by Amalfitano *et al.* (1998) suggested that deletion of additional early gene functions would result in reduced expression of late genes and increased transgene persistence. However, the eventual loss of transgene expression and the observed immune response showed that these second generation vectors still had limitations and required further modification.

1.4.4.2. Modifications to the E4 region.

Investigations of E4 gene product functions identified the Orf3 and Orf6 proteins as being essential, with either sufficient to provide an E4 function necessary for the normal viral lytic cycle (Bridge and Ketner, 1989; Huang and Hearing 1989a). Complementing cell lines have been developed which express E4-Orf6 (Brough *et al.*, 1996, Gao *et al.*, 1996) and these are capable of supporting the growth of vectors with the E4 region deleted. Studies by Gao *et al.* (1996) showed that deletion of the E4 region resulted in reduced liver toxicity and increased persistence of transgene expression when compared to first generation vectors in mouse models. An additional advantage of this E1/E4-deleted vector was the reduced risk of contamination by replication-competent virus, since there were no overlapping sequences between the cell's E4 gene and the vector E4 region to facilitate recombination. Lusky *et al.*, (1998) conducted an *in vivo* comparison of second generation vectors expressing *lacZ* or CFTR transgenes, that were isogenic for deletions in E1 and E3, with additional deletions in either the E2A or E4 genes. It was found that the E2A deletion had the most profound effect on late gene expression, however, no differences could be detected in the persistence of viral DNA in the liver and lungs of treated mice. These results led to the suggestion that viral antigens played a minor role in immune clearance of vector transduced cells.

Despite these findings recent publications present apparently conflicting data concerning the effect of E4 deletion on transgene persistence *in vivo*. Armentano *et al.* (1997) showed that retaining an entire E4 region could prolong transgene expression. They also showed that E4 gene products acted *in trans* to overcome down-regulation of expression of the transgene from the cytomegalovirus (CMV) intermediate-early promoter. E4-Orf6 alone was insufficient to overcome this effect but recent studies show that E4-Orf3 alone can achieve long-term transgene expression from the CMV promoter (Armentano *et al.* 1999), possibly by relieving its repression, as truncation of the CMV promoter abolishes the need for E4-Orf3. The earlier studies by Armentano *et al.* (1997) highlighted the importance of considering the vector background when choosing promoters to drive transgene expression. Although E4 was shown to have an effect on expression from the CMV promoter, no effect was seen on the persistence of transgene expression from similar vectors using the E1A promoter. These considerations were further emphasised by the studies of Dedieu *et*

al. (1997), who showed that E4 status had no effect on transgene persistence when using the Rous sarcoma virus LTR promoter.

Despite these apparent contradictions, E1/E4-deleted vectors have been shown to invoke reduced pathological responses in transduced mouse liver and therefore appear to be advantageous (Gao *et al.*, 1996, Dedieu *et al.*, 1997). Deletion of the E4 region also increases the transgene carrying capacity to about 11 kbp when engineered into a vector containing E1 and E3 deletions. There is also increasing evidence that E4 deletion may be necessary for safety reasons, namely the observations that E4-Orf1 can have transforming activity (Javier, 1994), that E4-Orf6 can block p53 activation (Dobner *et al.*, 1996) and cooperate with E1A to transform cells *in vitro* (Moore *et al.*, 1996).

1.4.4.3. Modifications to other regions.

Polypeptide IX is a minor capsid component involved in stabilising hexon interactions and is required for packaging of full-length genomes (Ghosh-Choudhury *et al.*, 1987). The polypeptide IX gene is within the E1B region and its previous deletion from vectors had placed a restriction on the size of transgene that could be inserted. Caravokyri and Leppard (1995) described the construction of a 293-based cell line expressing polypeptide IX. Use of this cell line allowed the construction of vectors with deletions in the IX gene that showed packaging capacity similar to that of IX⁺ vectors. Deletion of IX also allows insertion of up to 9.2 kbp of exogenous DNA into an E1/E3-deleted vector. Krougliak and Graham (1995) also used IX expressing cells to produce a vector deleted in E1, E3, E4 and pIX, resulting in a transgene carrying capacity of approximately 11 kbp.

Von Seggern *et al.* (1998) described the construction of a 293-based cell line expressing polypeptide IV, the constituent of the trimeric fibre protein. This cell line was capable of supporting the growth of a virus containing a *ts* mutation in the fibre protein and was subsequently used to produce fibre-less particles (Von Seggern *et al.* (1999).

1.4.5. Improving adenovirus vectors for gene therapy.

1.4.5.1. Regulation of the immune response.

Some of the major limitations to the use of adenovirus vectors in gene therapy are the vector-induced inflammation, the transient expression of transgenes, and the development of adenovirus-specific neutralising antibodies, which hinder repeat administration of the vector. Attempts to overcome these problems have manifested themselves in numerous approaches.

Reduction in both the humoral and cellular immune response has been achieved through the use of immunosuppressive reagents at the time of vector administration. Yang *et al.* (1996a) and Scaria *et al.* (1997) administered antibodies directed against molecules involved in the host immune response (CD4 and CD40 respectively), followed by administration of the vector. They showed that transgene expression was partially stabilised and that neutralising anti-Ad antibodies did not form, allowing repeat administration in both studies. Other studies have investigated blocking immune cell activation by blocking a co-stimulatory molecule necessary for the efficient stimulation of T cells, CTLA4. Kay *et al.* (1997) co-administered soluble CTLA4Ig, which is known to block co-stimulatory signals between T cells and antigen presenting cells, along with anti-CD40 antibody. These studies showed that use of both antibodies together, but neither one alone, led to maintenance of expression of human α_1 -AT in the livers of mice for over a year, and allowed effective repeat administration in 50% of animals. Similar studies by Jooss *et al.* (1998b) also demonstrated improved transgene persistence. While these results may be encouraging, systemic immune suppression may be unacceptable in the clinical setting. An alternative approach of expressing immunomodulatory proteins from the adenovirus vector alongside the principal transgene, may achieve targeted immune suppression and have fewer safety risks (Schowalter *et al.*, 1997a).

Adenovirus encodes its own immunomodulatory proteins, the products of the E3 transcription unit. Ilan *et al.* (1997) have demonstrated that expression of the E3 region from a CMV promoter reduces the anti-adenovirus humoral immune response. Intravenous administration of this vector to rats also resulted in a decrease in neutralising antibody formation, allowing repeat administration. The E3-encoded gp19K protein has been shown to block the ability of adenovirus-specific CTLs to recognise virus-infected cells *in vitro*, an effect it achieves by down-regulating the

expression of MHC class I molecules (Burgert and Kvist, 1987). Studies by Bruder *et al.* (1997) have shown that expression of gp19K from a vector leads to improved transgene expression in mouse liver and lungs. However a similar study by Schowalter *et al.* (1997b) failed to show any effect of gp19K expression on transgene persistence, although this could have been due to the use of a different promoter and mouse strain. Other studies have concentrated on blocking TNF α signalling, which is involved in triggering cytolysis and inflammation. Peng *et al.* (1999) showed that blocking TNF α activity in mouse liver and lung leads to prolonged transgene expression.

Another approach is to transiently deplete the numbers of macrophages before vector administration, as macrophages are responsible for removal of 90 % of transduced cells immediately after infection (Worgall *et al.*, 1997). The consequences of depletion of this cell type have been studied, using dichloromethane-bisphosphonate (Cl₂MBP), which is an immunosuppressant that depletes the numbers of liver macrophages. Studies by Wolff *et al.* (1997) showed a ten-fold increase in reporter gene levels after such treatment, plus the rate of removal of transduced cells was decreased.

As neutralising antibodies directed against one adenovirus serotype do not block infection by another serotype (Wolffhart, 1988; reviewed by Horwitz, 1996), one possible approach to achieving successful readministration is to alternate the serotype of the vector used. Mastrangeli *et al.* (1996) successfully administered an Ad5 vector to the lung, following prior immunisation with Ad4 or Ad30 virus. Similarly, successful transgene expression was achieved from an Ad5 vector following prior immunisation with Ad4 and Ad7 (Kass-Eisler *et al.*, 1996). Using a different approach, Roy *et al.* (1998) overcame the immunity to Ad5 by using a chimeric Ad5 vector containing the hexon protein from Ad12. This vector was successfully used to transduce the livers of mice previously immunised with Ad5.

1.4.5.2. Targeting adenovirus vectors to specific cell types.

Adenovirus vectors lack tissue-specificity due to the broad expression of the primary receptor, CAR. In contrast, transduction of some cell types is limited because of low expression of the CAR molecule. Therefore, the inability of adenovirus to

efficiently transduce certain tissues, coupled with its lack of tissue-specificity, has led to efforts to redirect adenovirus tropism.

Initial attempts at modifying tropism involved replacing the Ad5 fibre knob with the fibre knob from another adenovirus serotype. For example, switching the Ad5 fibre knob for the Ad3 fibre knob, which recognises a different cellular receptor (Stevenson *et al.*, 1995), allows the modified vector to transduce some cell types more efficiently (Stevenson *et al.*, 1997). Peptide ligands may also be inserted into the fibre protein to broaden the cell types that can be transduced by adenovirus. Wickham *et al.* (1996a) constructed a vector that contained a heparin-binding domain incorporated into the fibre protein, which targeted the virus to broadly expressed, heparin-containing cellular receptors.

Adenovirus tropism has also been modified by non-covalently attaching bispecific molecules to the virus. The bispecific molecule contains a first specificity for the virus and a second specificity for the cellular receptor. Wickham *et al.* (1996b) demonstrated that an adenovirus vector could be retargeted using a bispecific antibody recognising a FLAG (phenylalanine-leucine-alanine-glycine) peptide inserted into the Ad penton and the α_v integrin cellular receptor. This retargeting increased transduction of endothelial and smooth muscle cells that express α_v integrins. A drawback to this approach is that the adenovirus retains its native receptor-binding activity and is not truly retargeted. Douglas *et al.* (1996) overcame this problem and created a truly targeted adenovirus system. They achieved this by chemically conjugating folate to an anti-fibre antibody. This blocked native receptor binding and retargeted the vector to cells expressing the folate receptor. Similarly, when the antibody was conjugated to fibroblast growth factor 2 the vector was redirected to Kaposi's sarcoma cells expressing fibroblast growth factor receptors (Goldman *et al.*, 1997).

An alternative to modifying the tropism of the virus is to use a tissue-specific promoter. Larochelle *et al.* (1997) constructed vectors expressing a reporter gene under the control of a truncated creatine-kinase promoter/enhancer (MCK) which is specific for differentiated skeletal and cardiac muscle tissue. After injection of the vector to skeletal muscle, heart, lung, brain, kidney and liver of mice, reporter gene expression was only evident in the heart and skeletal muscle when compared to a

much broader spectrum of expression achieved by a similar vector containing the RSV-LTR promoter.

1.4.5.3. Increasing the transgene carrying capacity of adenovirus vectors.

The limited space available for insertion of heterologous sequences limits the use of adenoviral vectors for gene therapy. Complete deletion of the E1, E3 and E4 regions allows the insertion of approximately 11 kbp of exogenous DNA, which is not enough for some transgenes, e.g. the 14 kbp full-length dystrophin cDNA (Koenig *et al.*, 1987). Also the potential use of tissue specific promoter/enhancer sequences and the need to include immunomodulatory sequences, such as those from E3, further reduces capacity and indicates that current vector capacity will be insufficient. To solve these problems attempts have been made to generate adenovirus vectors with a large, often complete, deletion of viral coding sequences. These highly deleted viruses are often referred to as “gutless” or “guttled” vectors.

The only viral sequences required for efficient replication and packaging of viral genomes are the origins of replication (present in each ITR) and the packaging signal (bp 194 to 358 in Ad5). The remainder of the genome can be deleted and replaced with heterologous sequences, with the proteins necessary for virus growth being provided *in trans* either by a complementing cell line or a helper virus. Removal of all viral coding sequences clearly eliminates all expression of viral antigens in transduced cells and so would be expected to make the vector less immunogenic.

The first approaches toward creating vectors devoid of all viral coding sequences used replication-deficient helper viruses to provide the *trans*-complementation functions, with the helper virus being removed from the vector preparation subsequently by equilibrium density centrifugation. In a preliminary study by Mitani *et al.* (1995) it was shown that a vector deleted in L1, L2, VAI, VAIL and pTP could be constructed using *wt* adenovirus as the helper virus. Subsequent work by the same researchers demonstrated the construction of a vector devoid of all viral coding sequence, which was capable of expressing both β -galactosidase and full-length dystrophin *in vitro* (Kochanek *et al.*, (1996). The helper virus used in this study was rendered replication-defective by an E1-deletion and a partial deletion of the packaging sequence. This helper virus was removed from the vector preparation by caesium chloride density gradient centrifugation, resulting in vector preparations with

approximately 1 % contamination by helper virus. This vector was shown to be capable of β -galactosidase and dystrophin expression in muscle cells of *mdx* mice (Clemens *et al.*, 1996). However, dystrophin expression declined over 6 weeks post-vector administration, possibly due to an immune response directed against the reporter gene or to proteins expressed from the contaminating helper virus. Further studies by Chen *et al.* (1997) showed that stable expression of dystrophin in mouse muscle was possible if mice transgenic for *lacZ* were used, suggesting that an immune response directed at this reporter gene was responsible for loss of transgene expression. A similar vector was constructed by Fisher *et al.* (1996), encoding the human CFTR and grown using an E1-deleted helper virus. This group also suggested that more efficient ways of producing and purifying these vectors would be required before their use in gene therapy was feasible.

An alternative approach to provide helper functions without helper virus contamination of vector preparations involved the use of the Cre-*loxP* system from bacteriophage P1 (Parks *et al.* 1996; Hardy *et al.* 1997). *loxP* recombination sites were introduced on either side of the helper virus packaging signal. Upon infection of cells expressing the Cre recombinase enzyme the packaging signal is excised, rendering the helper virus incapable of packaging its genome. Thus the proteins encoded by the helper virus should be used exclusively to package coinfecting vector. Using a vector produced using this method, Schiedner *et al.* (1998) showed persistence of human α 1-AT expression in mouse liver when compared to that from a first generation vector. This expression was sustained for 40 weeks, at over 100-fold higher levels than was achieved from the first generation vector.

Despite these encouraging results, the production of “gutless” vectors is very inefficient, with only low titre obtained, which limits their potential use for *in vivo* human gene therapy applications. Studies by Hartigan-O'Connor *et al.* (1999) have shown “gutless” virions can be produced up to 50 times more efficiently if grown in cell lines coexpressing the adenovirus preterminal protein and DNA polymerase. Also the packaging efficiency of the genome is dependent on its size, with genomes 75 – 105 % of wild type length being packaged more efficiently (Parks and Graham, 1997). However, care must be taken when increasing the length of a vector genome to fall within these optimum limits. Sandig *et al.* (2000) showed that the nature of the so-called “stuffer DNA” used to increase genome size had an effect on the growth

characteristics of the vector and studies by Parks *et al.* (1999) demonstrated the possibility of CTL responses being directed against some “stuffer” sequences used.

1.4.6. Conclusions.

Progress in recombinant adenovirus technology has made this vector more and more attractive for *in vivo* gene transfer to many tissues. Highly deleted vectors can accommodate large transgenes associated with specific regulatory sequences that provide suitable regulation of transgene expression. The development of retargeting strategies has allowed adenovirus-mediated gene transfer to many new cell types. However, there are still major hurdles to be overcome before adenovirus vectors will achieve positive results in the clinic. The expression of residual viral late proteins and the potency of the immune response directed against them is one such problem.

1.5. Aims.

Second generation adenovirus vectors and highly-deleted vectors have their own advantages and disadvantages. Second generation vectors are easy to grow at high titres but are hampered by residual viral gene expression and low transgene carrying capacity. Highly deleted vectors have a large insert capacity and no residual viral gene expression. However, they are difficult to grow to high titres and purification away from the helper virus can be a problem. The work presented in this thesis was designed to address some of the limitations of current vector systems in order to build upon, and further develop past techniques for adenovirus vector production. The major aims were:

1. To create a plasmid construct containing the MLTU from human adenovirus type 5. The MLTU would be flanked by engineered PacI restriction sites, enabling it to be removed from the plasmid as a single fragment. This strategy would simplify any future manipulations of the MLTU and enable it to be cloned into alternative plasmid backbones. The first such manipulation would involve cloning the MLTU into an

episomal expression plasmid, which would be used subsequently to attempt to create a stable cell line.

2. To determine the scope of late protein expression from the cloned MLTU. This would be achieved by Western blotting using antibodies specific for Ad5 late proteins. Currently available antibodies were used, in addition to generating a new polyclonal antiserum with reactivity against virion proteins. Attempts were made to show the expression of at least one protein from each late gene.

3. To use the cloned MLTU construct to develop expression systems for adenovirus late proteins. Initially this would involve production of a stable cell line, with expression of the Ad5 late genes controlled by the major late promoter, which would be cloned as part of the MLTU. Other possibilities would then be explored such as the use of a regulated heterologous promoter to control late gene expression.

The overall aim of this work was to move towards the creation of a cell line capable of complementing the growth of an adenovirus vector with late gene deficiencies.

Chapter 2 : Materials and Methods.

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2.1. Materials.

2.1.1. Common buffers and solutions.

5 X SDS-PAGE loading buffer: 2 % (w/v) SDS, 5 % (v/v) β -mercaptoethanol, 10 % (v/v) glycerol, 0.01 % (v/v) bromophenol blue.

chloroform/iso-amyl alcohol: 96 % (v/v) chloroform, 4 % (v/v) iso-amyl alcohol

50 X Denhardt's solution: 1 % (w/v) Ficoll, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) BSA.

LB: 1 % (w/v) bactotryptone, 1 % (w/v) NaCl, 0.5% (w/v) yeast extract

LB agar: LB containing 1.5 % (w/v) bacto-agar

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM Na_2HPO_4 , 1.4 mM KH_2PO_4

PBS/glycerol: 40 % (v/v) PBS, 60 % (v/v) glycerol

Phenol/chloroform: 50 % (v/v) Tris buffered phenol, 48 % (v/v) chloroform, 2 % (v/v) iso-amyl alcohol

TBE: 89 mM Tris. base, 89 mM boric acid, 1 mM ethylenediaminetetra-acetic acid (EDTA) pH 8.0

TD: 25 mM Tris.HCl pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4

TE: 10 mM Tris.HCl pH 8.0, 1 mM EDTA

TFB I: 100 mM RbCl, 50 mM MnCl_2 , 10 mM CaCl_2 , 30 mM potassium acetate, 19 % (w/v) glycerol, pH 5.8

TFB II: 10 mM 3-[N-Morpholino]propanesulphonic acid (MOPS), 10 mM RbCl, 75 mM CaCl_2 , 19 % (w/v) glycerol, pH 7.0

TNE: 10 mM Tris.HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0

TS: 25 mM Tris.HCl pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 0.9 mM CaCl_2 , 1 mM MgCl_2

10 X SSC: 87.65 g/l NaCl, 44.1 g/l sodium citrate, pH 7.0

2.1.2. Bacterial strains.

<i>E. coli</i> strain	Phenotype
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^fZΔM15 Tn10 (Tet^r)</i>]. (Used for all cloning steps).

2.1.3. Adenovirus strains.

Virus strain	Phenotype
<i>wt300</i>	Wild type (Jones and Shenk, 1979).

2.1.4. Cell lines.

Cell line	Origin
293	Adenovirus-transformed human embryonic kidney (Graham et al, 1977)
Hela	Human cervical epitheloid carcinoma (Scherer <i>et al</i> , 1953).
A549	Human lung carcinoma (Giard <i>et al</i> , 1973).

2.1.5. Antibodies and conjugates.

Primary antibody		Dilution used	
Name / protein(s) recognised	Type / origin	Western	I.F
AbBF1 II, III	Mouse polyclonal (from B. Flanagan, University of Warwick).	1/500	
AbR1/89 III, IV, L4 100k	Rabbit polyclonal (from V. Mautner, University of Birmingham).	1/1000	
Anti-DBP 72K DBP	Rabbit polyclonal (from E. Harfst, University of Warwick).	1/1000	
Anti-100K	Mouse monoclonal (from W.C.	1/1000	1/1000

L4 100K	Russell, University of St Andrews).		
Anti-IVa2 IVa2	Rabbit polyclonal (from S. Brey, PhD thesis, University of Warwick, 1999).	1/1000	
Anti – pIX pIX	Rabbit polyclonal (Caravokyri and Leppard, 1995).	1/1000	
Anti-L1 52/55K L1 52/55K	Rabbit polyclonal (from A. Arslanoglu, PhD thesis, University of Warwick, 1999).	1/1000	
Secondary antibody			
Rabbit Ig	Biotinylated / goat	1/1000	
Mouse Ig	Biotinylated / goat	1/1000	
Rabbit Ig	HRP conjugated / donkey	1/1000	
Mouse Ig	HRP conjugate / sheep	1/1000	
Rabbit Ig	Rhodamine conjugate / goat		1/200
Rabbit Ig	Fluorescein conjugate / goat		1/200
Mouse Ig	Fluorescein conjugate / goat		1/200
Conjugate			
	Streptavidin-biotinylated HRP complex	1/500	

2.1.6. Oligonucleotides.

All oligonucleotides were obtained from Gibco BRL and are shown 5' to 3'. Annealing sites for primers are given in the text when they arise.

2.1.6.1. PCR primers.

Primer #1: TCCTCCTGTTCTCCTGTCCATC

Primer #2: ATCATCGCTGAGGAGACCAC

Primer #3: GATCTGTTAATTAAGCTATGTGGTGGTGGGGCTATAC

Primer #4: CATAGCTTAATTAACAGATCACCGTACCTTAATC

2.1.6.2. Sequencing primers.

SEQ-D: CTGTGTACTCTGTGTGTTGG

2.1.6.3. Adaptors.

NdeI-PacI adaptor: TATTAATTAA

BamHI-PacI adaptor: GATCTTAATTAA

EcoRI-PmeI adaptor: AATTGTTTAAAC

2.2. Suppliers.

All chemicals of analytical or molecular biology grade were supplied by Sigma (Poole, Dorset, UK) or BDH Laboratory supplies (Merck Ltd, Poole, Dorset, UK) unless otherwise stated below.

Amersham Pharmacia Biotech (Bucks., UK).

Biotinylated goat α -mouse IgG, Biotinylated goat α -rabbit IgG, streptavidin-biotinylated horseradish peroxidase complex, α -³²P-dCTP, Hybond N and Hybond C membranes, SDS-PAGE molecular weight markers, Sephadex[®] G50 medium, Hyperfilm[™] ECL, ECL[™] Western blotting detection reagents, dATP, dCTP, dTTP, dGTP.

Becton-Dickinson (Cockeysville, USA).

Yeast extract.

Biorad (Hemel Hempstead, Herts., UK).

Acrylamide, N.N'-methylene-bis-acrylamide, ammonium persulphate, 4-chloronaphthol, electrophoresis grade sodium dodecyl sulphate (SDS), Biorad protein assay reagent.

BOC (Surrey, UK).

CO₂, dry ice.

Boehringer Mannheim UK (East Sussex, UK).

Hygromycin B.

Calbiochem (Nottingham, UK).

Goat α -mouse IgG fluorescein isothiocyanate (FITC)-conjugate, goat α -rabbit IgG rhodamine conjugate, goat α -rabbit IgG fluorescein isothiocyanate (FITC)-conjugate.

Difco Laboratories (Hants., UK).

Bacto-agar, bactotryptone, Noble agar.

Fisher Scientific (Loughborough, UK).

Caesium chloride, sodium dodecyl sulphate (SDS).

Fuji Photo Film Co. Ltd. (Dusseldorf, Germany).

Super RX x-ray film.

Gibco BRL Life Technologies Ltd. (Renfrewshire, Scotland).

Restriction enzymes, 1kb DNA ladder, calf intestinal alkaline phosphatase (CIAP), Taq DNA polymerase, DNA polymerase I large (Klenow) fragment, T4 DNA ligase, T4 RNA ligase, T4 DNA polymerase, Dulbecco's modified Eagles medium (DMEM), newborn calf serum (NCS), foetal calf serum (FCS), trypsin, versene.

Kodak (London, UK).

X-ray developing fluid, X-ray fixative.

New England Biolabs (Hearts., UK).

Restriction enzymes, Vent[®] DNA polymerase.

Premiere Beverages (Stafford, UK).

Dried milk powder (Marvel).

Promega UK (Southampton, UK).

Wizard[®] PureFection plasmid purification kit.

Qiagen (Hilden, Germany).

Qiaprep miniprep reagents.

University of Warwick media preparation service.

Sterile PBS, distilled H₂O.

Vector Laboratories (Burlingame, CA).

Vectashield[®] mounting medium.

Whatman International (Maidstone Kent).

3MM chromatography paper.

2.3. Methods.

2.3.1. DNA manipulations.

2.3.1.1. Restriction enzyme digestion.

All DNA was digested in the buffers supplied by the manufacturer at the recommended temperature, typically in a volume of 20 - 100 µl. Digestion of DNA with multiple enzymes was performed in the buffer which gave optimum activity of all enzymes. Reactions were stopped by the addition of EDTA to 25 mM or heat inactivation where appropriate.

2.3.1.2. Dephosphorylation of DNA.

Dephosphorylation reactions were performed in REact3™ (50 mM Tris.HCl pH 8.0, 10 mM MgCl₂, 100 mM NaCl), using calf intestinal alkaline phosphatase (CIAP). DNA fragments with protruding 5' termini were incubated at 37°C for 30 min with 1 unit of CIAP, followed by a repeat incubation with a further aliquot of CIAP. DNA fragments with recessed 5' termini or non-cohesive ends were incubated at 37 °C for 15 min, 55 °C for 15 min with 1 unit of CIAP, followed by repeat incubations with a further aliquot of CIAP. All reactions were stopped by heat inactivation (75 °C, 15 min) in the presence of 25 mM EDTA. Electrophoresis and electroelution was used to purify the dephosphorylated DNA prior to its use in any cloning steps.

2.3.1.3. End filling of 5' overhangs (Klenow filling).

DNA fragments with extended 5' termini were converted to non-cohesive ends using the Klenow fragment of *E.coli* DNA polymerase I. DNA was incubated with 2 units of enzyme at 22 °C for 20 min in 50 mM Tris.HCl pH 8.0, 10 mM MgCl₂, 50 mM NaCl. The reaction was stopped by heat inactivation (70 °C, 5 min).

2.3.1.4. Removal of 3' overhangs.

DNA fragments with extended 3' termini were converted to non-cohesive ends using T4 DNA polymerase. DNA was incubated with 2 units of enzyme at 37 °C for 5 min in 33 mM Tris-HCl pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 100 µM dATP, 100 µM dTTP, 100 µM dCTP, 100 µM dGTP. The reaction was stopped by heat inactivation (75 °C, 10 min).

2.3.1.5. Ligation of DNA molecules.

DNA fragments with cohesive ends were ligated for 16 hrs at 15 °C, using 2 units of T4 DNA ligase in the supplied buffer (50 mM Tris.HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol (DTT), 5 % (w/v) polyethylene glycol (PEG) 8000). DNA fragments with non-cohesive ends were ligated for 16 hrs at 22 °C, using 10 units of enzyme in the buffer described above. Typically a 5 fold molar excess of insert over vector DNA fragments was used.

2.3.1.6. Gel electrophoresis of DNA molecules.

DNA fragments were separated on type I agarose (0.6 – 1.4 % w/v), TBE gels, containing 0.5 mg/ml ethidium bromide. Samples were loaded in 30 % glycerol, 0.04 % bromophenol blue. Electrophoresis was performed in TBE at 70 mA until the required degree of separation was achieved. Bands were then visualised by transillumination with UV light and photographed using computerised gel imaging equipment (Prior Laboratory supplies, UK). DNA fragment sizes were estimated by comparison to a DNA ladder of known fragment size (1 kb ladder, GibcoBRL).

2.3.1.7. Electroelution of DNA molecules from agarose gels.

The DNA to be purified was excised from the gel and eluted into sealed dialysis tubing (molecular weight cut-off, 16,000) containing 500 µl TBE by electrophoresis in 0.5 X TBE at 100 V for 1 hr. The eluate was then phenol / chloroform extracted twice, followed by ethanol precipitation.

2.3.1.8. Phenol/chloroform extraction.

An equal volume of phenol / chloroform was added to the DNA solution and mixed by repeated inversion. Phase separation was achieved by centrifugation at 10,000 x gravity (g) for 2 min; the lighter aqueous phase was removed to a clean tube.

2.3.1.9. Ethanol precipitation.

DNA was precipitated from aqueous solution by addition of sodium acetate to 300 mM, plus 2 volumes of ethanol. This was placed at -20 °C for 1 hr, after which the DNA was pelleted by centrifugation at 18,000 x g for 15 min, washed with 70 % (v/v) ethanol and re-centrifuged for 5 min. The pellet was dried briefly in a vacuum dessicator and resuspended in 10 mM Tris.HCl pH 7.5.

2.3.1.10. Spectrophotometric quantification of DNA.

The concentration and purity of DNA in aqueous solution was determined by absorbancies at 260 nm and 280 nm. An absorbance of 1.0 at 260 nm is equal to 50 µg/ml for double stranded DNA. Purity was assessed by the ratio of absorbance at 260 nm to that at 280 nm, a ratio of 1.7 – 1.8 indicating high purity.

2.3.1.11. Automated DNA sequencing.

Automated DNA sequencing was performed by Mrs L. Ward, Department of Biological Sciences, University of Warwick, using an Applied Biosystems sequencer (model 373a).

2.3.1.12. Polymerase chain reaction (PCR) (after Mullis et al. 1986).

Target DNA was amplified by PCR; all reactions were optimised but a typical 50 µl reaction mix would consist of 100 ng target DNA, 1 mM each of dATP, dCTP, dGTP, dTTP, required concentration of MgSO₄, 1 unit of Vent[®]DNA polymerase, in the buffer supplied by the manufacturer. Reaction conditions were:

94 °C, 1 minute; 60 °C, 1 minute; 74 °C, 3 min (19 cycles), and then

94 °C, 1 minute, 60 °C; 1 minute, 74 °C; 7 min (1 cycle), followed by a 4 °C soak until the sample could be removed for processing.

2.3.2. Bacteriological techniques.

2.3.2.1. Culture and storage of bacteria.

E.coli XL1-Blue were cultured in LB containing 15 µg/ml tetracycline at 37 °C with shaking. Transformed bacteria were grown on LB agar plates or as liquid cultures, both containing 100 µg/ml ampicillin (LBamp) and incubated at 37 °C with shaking. Bacteria containing larger plasmids (20-40 kb) were cultured at 30 °C to allow efficient plasmid replication.

Cultures were maintained in long-term storage as a 50 % (v/v) mix of glycerol and exponential phase liquid culture, at -80 °C.

2.3.2.2. Preparation and transformation of competent bacteria.

5 ml of LB was inoculated with a single colony of *E. coli* strain XL1-Blue and cultured to stationary phase at 37 °C. 50 µl of this was used to inoculate 25 ml of LB which was cultured to OD₅₅₀ 0.3 – 0.4. This was split equally between two flasks, each containing 250 ml of LB, and cultured to OD₅₅₀ 0.4 – 0.5. The cultures were cooled on ice for 15 min then the cells were harvested by centrifugation (1,300 x g, 10 min, at 4 °C). Pellets were resuspended gently in 100 ml of ice-cold TFB I and re-centrifuged. The supernatant was discarded and the pellet resuspended in 10 ml of ice-cold TFB II. 0.5 ml aliquots were rapidly frozen in a dry ice/ethanol bath and stored at -70 °C.

To transform competent bacteria, DNA (10 ng control plasmid or 15 µl ligation reaction) was diluted in 100 µl of sterile distilled water and placed on ice. Competent bacteria were thawed rapidly and 150 µl added to the DNA solution, which was left on ice for 1 hr, subjected to heat-shock (42 °C, 2 min) then placed on ice for 5 min. 300 µl of LB was then added and the cells incubated at 37 °C for 30 min prior to

plating on LB agar plates containing 100 µg/ml ampicillin, which were then incubated for 16 hrs at 37°C.

2.3.2.3. Small scale preparation of plasmid DNA.

Plasmid DNA was isolated from 1.5 ml of bacterial culture using a Qiagen Miniprep plasmid purification kit, following the manufacturer's instructions. This DNA was suitable for restriction enzyme analysis and cloning.

2.3.2.4. Large scale preparation of plasmid DNA.

A single bacterial colony containing the plasmid of interest was used to inoculate 1.5 ml of LBamp and cultured to stationary phase. 50 µl of this was used to inoculate 25 ml of LBamp, which was cultured to OD₆₀₀ of 0.6. This culture was then used to inoculate 500 ml of LBamp and cultured to OD₆₀₀ of 0.4. Chloramphenicol at 100 µg/ml was then added to amplify the plasmid, and the culture was incubated for a further 16 hrs. All incubations were carried out at 37 °C unless stated otherwise in the relevant results chapter.

Cells were collected by centrifugation (1,600 x g, 30 min, 4 °C) and resuspended in 10 ml of cold 25 % sucrose, 50 mM Tris.HCl pH 8.0. 4 ml of cold 0.25 M EDTA was added, mixed gently and placed on ice for 5 min. 20 mg of lysozyme dissolved in 2 ml of TNE was then added, mixed gently and placed on ice for 15 min. Following this, 16 ml of cold Triton Lysis mixture (0.1 % (v/v) Triton X-100, 62.5 mM EDTA, 50 mM Tris.HCl pH 8.0) was added, mixed gently and placed on ice for a further 5 min. Cellular debris was then removed by centrifugation (35,000 x g, 40 min, 4 °C). The supernatant was then extracted by mixing vigorously with 7 ml Tris-buffered phenol and 1 ml chloroform/iso-amyl alcohol, allowed to stand for 10 min and then centrifuged (18,000 x g, 10 min, 4 °C). The aqueous phase was removed and further extracted by the addition of 20 ml chloroform/iso-amyl alcohol, vigorous mixing and centrifugation as before. The aqueous phase was removed and DNA precipitated by the addition of 2 volumes of 95 % (v/v) ethanol at -70 °C for 1 hr. The DNA was pelleted by centrifugation (18,000 x g, 20 min, 4 °C) and dried in a vacuum dessicator for 15 min. The pellet was resuspended in 4.5 ml TNE , to which was added 4.72 g

CsCl and 320 µl ethidium bromide (10 mg/ml). Plasmid DNA was purified by equilibrium density gradient centrifugation in a Beckman Vti65 rotor (200,000 x g, 16 hrs, 20°C). Ethidium bromide was removed from the DNA by repeated extraction with an equal volume of NaCl-saturated isopropanol. 3 volumes of TNE were then added and the DNA precipitated at -70 °C using 95 % (v/v) ethanol. Plasmid DNA was recovered by centrifugation (15,900 x g, 30 min, 4 °C), washed in 70 % (v/v) ethanol, then dried and resuspended in 0.5 ml 10 mM Tris.HCl pH 8.5, containing 10 µg RNaseA.

Alternatively, plasmids were purified using the Purefection® Plasmid DNA Purification kit (Promega), following manufacturer's instructions.

2.3.3. Tissue culture techniques.

2.3.3.1 Maintenance of cell lines.

All cell lines were cultured in 90 mm γ-irradiated tissue culture dishes at 37 °C in a 5 % CO₂ atmosphere and passaged when confluent. 293 cells (and derivatives) were maintained in DMEM / 10 % NCS (v/v), HeLa and A549 cells were maintained in DMEM / 10 % FCS. Cells were passaged as follows: monolayers were washed with 0.02 % (v/v) versene, then cells detached by adding 2.5 ml of 0.02 % (v/v) versene containing 1.25 mg trypsin, for 3-5 min. Cells were removed into 0.5 ml NCS and centrifuged at 600 x g for 4 min. The pellet was resuspended in the appropriate medium and cells seeded at the required density.

2.3.3.2. Long term storage of mammalian cells in liquid nitrogen.

Sub-confluent 90 mm dishes of cells were trypsinised for passage and the cell pellet resuspended in 1 ml of 92 % (v/v) NCS, 8 % (v/v) dimethyl sulphoxide (DMSO). The cell suspension was frozen in 0.5 ml aliquots by cooling slowly to -70 °C overnight. Frozen vials were subsequently transferred to liquid nitrogen.

2.3.3.3. Recovery of frozen cell stocks from liquid nitrogen storage.

Cells were thawed rapidly at 37 °C then transferred to 10 ml of pre-warmed growth medium in a 90 mm dish. The medium was replaced the following day to remove residual DMSO.

2.3.3.4. Transfection of mammalian cells with dsDNA.

2.3.3.4.1 Calcium phosphate transfection.

Cells to be transfected were grown to the required density (usually 70 % confluency) in 60 mm tissue culture dishes. The calcium phosphate / DNA precipitate was prepared as follows : 0.5 ml of mix A (250 mM CaCl₂) containing the DNA to be precipitated was added dropwise to 0.5 ml of mix B (0.06 mM Na₂HPO₄, 10 mM KCl, 270 mM NaCl, 0.2 % (w/v) glucose, 1 % (w/v) N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), pH 7.08 – 7.12) whilst bubbling air through mix B. This was left at room temperature for 30 min then added dropwise to the cell cultures, 0.5 ml per 60 mm dish containing 5 ml growth medium. Cells were incubated at 37 °C for 4 hrs and the medium replaced. Cells were subsequently incubated at 37 °C / 5 % CO₂ for the desired period.

2.3.3.4.2. Liposome mediated transfection.

Cells were transfected with LIPOFECTAMINE™, LIPOFECTIN™ and LIPOFECTACE™ (Gibco BRL, Renfrewshire, Scotland) according to manufacturer's instructions.

2.3.4. Virological techniques.

2.3.4.1. Virus infection of mammalian cells.

Infections were initiated by removing the growth medium and adding an amount of virus calculated to give the desired multiplicity of infection, diluted in DMEM without serum. Cells were incubated at 37 °C and rocked every 15 min. After 1 hr, full growth medium containing serum was added and the cells incubated at 37 °C for the time appropriate to the experiment.

2.3.4.2. Large scale preparation of virus stocks and DNA.

90 % confluent dishes of cells were infected with virus (m.o.i. of 2 pfu/cell) and harvested at full cytopathic effect. Cells were collected by centrifugation (500 x g, 5 min) and resuspended in cold 0.1 M Tris.HCl pH 8.0, to a final volume of 5 ml for up to 4 dishes. This suspension was sonicated on ice using a Jencons sonicator with a 3 mm tip, using two sets of ten, one second, pulses separated by 30 seconds. Cell debris was removed by centrifugation (5,600 x g, 10 min, 4 °C) and the supernatant layered over a two-step CsCl gradient (2 ml 1.4 g/ml CsCl, 3 ml 1.25 g/ml CsCl, both in TD). Gradients were centrifuged (150,000 x g, 1 hr, 15 °C) in a Beckman SW 41 rotor and the virus band collected by puncturing the tube and collecting the relevant part of the flow-through. This was then diluted with 1.35 g/ml CsCl / TD and centrifuged to equilibrium (150,000 x g, 16 hrs, 15 °C) in a Beckman SW 50.1 rotor. The virus band was collected as before and either used to prepare a purified virus stock or viral DNA:

(i). Purified virus stock.

A small sample of the collected virus was diluted 1 in 500 with TE / 0.1 % (w/v) SDS and the optical density measured. Virion concentration was determined using the formula, A_{260} of 1.0 = 10^{12} virions/ml. The remaining virion stock was then diluted five fold with stabilising buffer (0.1 % (w/v) BSA, 50 % (v/v) glycerol, 10 mM Tris.HCL pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$) and stored at -20 °C.

(ii). Viral DNA.

Collected virus was diluted with two volumes of H_2O and precipitated with 2 volumes of ethanol at -70 °C for 30 min. Virions were pelleted by centrifugation (7,600 x g, 20 min, 4 °C), dried briefly and resuspended in 2 ml TNE containing 120 µl 10 % (w/v) SDS, 40 µl 250 mM EDTA and 200 µg proteinase K. This was incubated at 37 °C for 1 hr, then extracted twice with an equal volume of phenol/chloroform. The aqueous phase was collected and NaCl added to a final concentration of 0.1 M. Viral DNA was precipitated by addition of 2 volumes of ethanol at -70 °C for 30 min. DNA was pelleted by centrifugation (15,900 x g, 30 min, 4 °C), washed in 70 % ethanol, dried and resuspended in 0.5 ml TE pH 8.0.

2.3.5. Analysis of protein expression.

2.3.5.1. Extraction of total cellular protein from mammalian cells.

Cells and medium from 60 mm dishes were collected and the cells harvested by centrifugation (500 x g, 5 min). Pellets were resuspended in a small volume (100 – 200 µl) of 1 X SDS-PAGE loading buffer containing 1 mM phenylmethylsulphonyl fluoride, vortexed and placed on ice for 15 min. Cell debris was removed by centrifugation (18,000 X g, 30 min) and samples stored at –20 °C.

2.3.5.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Protein samples were denatured and reduced by boiling in SDS-PAGE loading buffer for 5 min. Proteins were separated by electrophoresis in discontinuous SDS-polyacrylamide gels according to the method of Laemmli (1970). Gels consisted of a 5 % stacking gel (120 mM Tris.HCl pH 6.8, 4.57 % (w/v) acrylamide, 0.12 % (w/w) N.N'-methylene-bis-acrylamide, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.3 µl/ml TEMED) and a 10 % resolving gel (430 mM Tris.HCl pH 8.8, 10 % (w/v) acrylamide, 0.27 % (w/v) N.N'-methylene-bis-acrylamide, 0.1 % (w/v) SDS, 0.07 % (w/v) ammonium persulphate and 0.2 µl/ml TEMED). Electrophoresis was performed for 2-3 hrs at 240 V in running buffer (50 mM Tris base, 380 mM glycine, 0.1 % (w/v) SDS) using the Gibco BRL vertical gel electrophoresis system. Alternatively the Bio-Rad mini-PROTEAN II electrophoresis system was used according to the manufacturer's instructions.

2.3.5.3. Western blotting.

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane according to the method of Towbin et al. (1979). The gel and membrane were equilibrated in transfer buffer (25 mM Tris.HCl, 192 mM glycine, 20 % methanol) for 30 min. Proteins were transferred to the membrane by electroblotting using a Bio-Rad Trans-Blot™ apparatus at 70 V for 3 hrs. Alternatively, electroblotting of mini gels was performed in the Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell, at 100 V for 1 hr. The membrane was blocked in PBS / 5 % (w/v) milk powder for 1 hr at

room temperature or overnight at 4°C, then probed with antibodies (see 2.1.5 for dilutions used). The membrane was incubated with primary antibody for 2 hrs, followed by a 1 hr incubation with secondary antibody and a 1 hr incubation with enzyme conjugate (if required). All antibodies were diluted in PBS and incubations performed at room temperature with vigorous shaking. Between each incubation membranes were washed four times (5 min per wash) in PBS / 0.1 % (v/v) Tween 20. Following the final wash membranes were washed in PBS (2 x 5 minute washes) prior to developing. Membranes were developed by addition of a freshly made mixture of 20 ml of methanol containing 60 mg 4-chloro-naphthol (4CN) and 100 ml PBS containing 60 µl H₂O₂ (100 vol). Membranes were washed in distilled water after bands reached the desired intensity. Alternatively, ECL™ Western blotting detection reagents (Amersham) were used according to the manufacturer's instructions.

2.3.5.4. Fluorescence microscopy of cell monolayers.

Expression of GFP in eukaryotic cell cultures was observed by growing cells on glass coverslips in 6 well tissue culture dishes. Coverslips were washed twice in PBS, mounted in Vectashield® mounting medium, then viewed and photographed using a Nikon epifluorescence microscope, using a UV filter (B2-H, Nikon) with an excitation filter from 450-490 nm.

2.3.5.5. Immunofluorescence microscopy.

Cells grown on coverslips were washed twice in PBS, fixed for 15 min in methanol (at -20 °C), washed again and then stored immersed in PBS at 4 °C). Before analysis coverslips were incubated in PBS / 1 % BSA for 1 hr and all reagents used subsequently were diluted in this solution. To detect specific antigens, coverslips were incubated with the relevant primary antibody for 1 hr, followed by three washes in PBS. Bound primary antibody was then detected by incubating coverslips for 1 hr with species-specific secondary antibody conjugates (see section 2.1.5 for details and dilutions used), and then washed as before. If staining of nuclei was required, 4,6-diamidino-2-phenylindole (DAPI) was added to the final PBS wash at 1 µg/ml. Coverslips were mounted in Vectashield® mounting medium, then viewed and photographed using a Nikon epifluorescence microscope.

2.3.5.6. Protein assay.

The Bio-Rad protein assay™ was used according to the manufacturer's instructions using BSA as a standard for determination of protein concentrations.

2.3.5.7. Detection of β -galactosidase activity in mammalian cells.

Cell monolayers grown on coverslips were washed twice in PBS and fixed (100 mM Na_2HPO_4 , 100 mM NaH_2PO_4 , 0.1 mM MgCl_2 , 0.225 % (v/v) glutaraldehyde) for 15 min. Cells were washed twice before the addition of X-gal staining solution (PBS containing 10 mg/ml (w/v) X-gal, 4 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 4 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 2 mM MgCl_2) and left overnight at 37 °C. Staining solution was then removed, followed by one wash in PBS and coverslips mounted in PBS/glycerol. Cells were photographed using a Nikon phase-contrast microscope.

2.3.6. DNA extraction from mammalian cells.

2.3.6.1. Extraction of total cellular DNA.

Cells grown as monolayers in 90 mm dishes were washed from the dish in PBS and harvested by centrifugation (500 x g, 5 min). Pellets were resuspended in 2 ml RIPA buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl, 1 % (w/v) sodium deoxycholate, 1 % (v/v) Triton-X100, 0.1 % (w/v) SDS), left on ice for 30 min and vortexed at five minute intervals. This was then extracted twice with an equal volume of phenol/chloroform and once with chloroform/iso-amly alcohol alone. The aqueous phase was removed, sodium acetate added to 0.3 M and DNA precipitated by adding 2 volumes of 95 % (v/v) ethanol at -70 °C for 1 hr. DNA was pelleted by centrifugation (15,900 x g, 30 min, 4 °C), washed in 70 % (v/v) ethanol, dried briefly and resuspended in 200 μl TE, containing 2 μg RNaseA.

2.3.7. Detection of specific DNA by Southern blotting.

2.3.7.1. Generating a radioactive probe by nick translation.

Probes for use in Southern Blotting were generated by nick translation. The reaction consisted of 30 μ Ci α -³²P-dCTP (specific activity 3000 Ci/mmol), 200ng target DNA, 0.1 mg/ml DNase I, 1 unit DNA polymerase I, 20 mM each of dATP, dGTP and dTTP, 14 mM Tris.HCl pH 7.4, 7 mM MgCl₂, 50 mM NaCl and 2 mM DTT. The reaction was incubated at 16 °C for 3 hrs and the probe separated from unincorporated nucleotides. This was achieved using a Sephadex[®] G50 column of 10 ml bed volume in TNE/0.1 % (w/v) SDS. The sample was loaded in an equal volume of 30 % (v/v) glycerol, 1 % (w/v) SDS, 50 mM Tris.HCl pH 8.0, 0.01 % (w/v) bromophenol blue, and 0.3 ml flow-through fractions collected. The fractions containing probe were pooled and denatured by boiling for 10 min then placed immediately on ice, prior to use.

2.3.7.2. Southern transfer of DNA to nitrocellulose (after Southern, 1975).

DNA was separated on agarose gels and exposed to short wave UV light for 5 min. Gels were then soaked in 1.5 M NaCl, 0.5 M NaOH for 30 min, 2 M NaCl, 1 M Tris.HCl pH 5.5 for 30 min, then placed in 2 X SSC. DNA was transferred to nylon membrane overnight in 10 X SSC by capillary action.

DNA was fixed onto the membrane using a Stratalinker[®]UV Crosslinker (Stratagene, CA). The membrane was then soaked in prehybridisation fluid (50 mM Tris.HCl 7.5, 0.5 % (w/v) SDS, 1 mM EDTA, 3 X SSC, 100 μ g/ml denatured salmon sperm DNA, 1 X Denhardt's solution) for 4 hrs at 64 °C then soaked for 16 hrs at 64 °C in prehybridisation fluid containing radioactive DNA probe.

The membrane was then washed in 2 X SSC, 0.5 % (w/v) SDS for 5 min at room temperature, 2 X SSC, 0.1 % (w/v) SDS for 15 min at room temperature, 0.1 X SSC, 0.5 % (w/v) SDS for 2 hrs at 64 °C and 0.1 X SSC, 0.5 % (w/v) SDS for 30 min at 64 °C. The membrane was then blotted dry, wrapped in cling-film and exposed to x-ray film at -70 °C. Intensifying screens were used to enhance band intensity.

**Chapter 3 : Construction of an episomal
expression plasmid containing the Major Late
Transcription Unit (MLTU) of Ad5.**

Chapter 3 : Construction of an episomal expression plasmid containing the Major Late Transcription Unit (MLTU) of Ad5.

3.1. Introduction.

Conventional adenovirus gene delivery vectors are based on replacement of early regions of the viral genome with an expression cassette coding for a gene of interest (discussed in detail in section 1.4). These vectors have two major drawbacks which limit their effectiveness. First, they have a capacity for insertion of foreign sequences which is limited to around 8 –10 kb. Second, despite deletion of the E1 region these vectors still express low levels of immunogenic late proteins. This leads to an immune response and elimination of transduced cells when used *in vivo*. Gutted adenovirus vectors overcome these drawbacks but introduce another problem, namely that the helper virus used in the preparation of vector can contaminate the stock. The ideal solution to these problems would be a vector with properties between these two extremes, having a high insert capacity and reduced expression of immunogenic proteins but not requiring helper virus for growth. Developing such vectors forms the basis for the research presented in this thesis.

3.2. Aims.

The aim of this work was to construct a plasmid containing the major late transcription unit (MLTU) of Ad5, with expression driven by the major late promoter (MLP). This construct would be flanked by *PacI* sites to make any further manipulations more straightforward. Ultimately an expression plasmid based upon an Epstein-Barr virus (EBV) replicon would be constructed which would be capable of episomal maintenance in eukaryotic cells.

3.3. Episomal maintenance of EBV-derived expression vectors.

The first step towards constructing an adenovirus deleted in one or more essential functions is to create a complementing cell line capable of expressing the genes

intended for deletion. An expression plasmid containing an Epstein-Barr virus replicon was chosen to be used to create a complementing cell line expressing the Ad5 MLTU. This approach has been used previously to construct a cell line capable of complementing a pIX-deleted virus (Caravokyri and Leppard, 1995).

Epstein-Barr virus (EBV) is a gammaherpesvirus that establishes a latent infection in B-lymphocytes. EBV is widespread in all human populations, with the great majority having antibodies to the virus (reviewed by Rickinson and Kieff, 1996). Infection may contribute to the initiation and progression of several human neoplasias including Burkitt's lymphoma and nasopharyngeal carcinoma (reviewed by Zur Hausen, 1981; Kieff, 1996). During latency the ~170 kb double stranded DNA genome replicates as a circular episome that divides synchronously with the cellular genome (Adams, 1987; Yates and Guan, 1991). The replication and stable maintenance of latent genomes requires only two viral elements: the protein, Epstein-Barr virus nuclear antigen 1 (EBNA-1, Hearing *et al.*, 1984) and the latent replication origin, oriP (Yates *et al.*, 1984, 1985).

EBNA-1 binds to clusters of sites in two regions of oriP which are both essential and together sufficient for viral DNA replication (Rawlins *et al.*, 1985). One region, termed the dyad symmetry region, spans ~120 bp and contains four EBNA-1 binding sites. The other known functional element of oriP is a 20-member family of 30 bp tandem repeats, with each repeat containing an EBNA-1 binding site. In addition to its well known function in DNA replication, other studies have suggested that EBNA-1 might also facilitate the retention of viral genomes (Krysan and Calos, 1993; Middleton and Sugden, 1992,1994). EBNA-1 has also been shown to bind to metaphase chromosomes (Marechal *et al.*, 1999), suggesting it may play a role in anchoring viral genomes to cellular chromosomes, thus ensuring proper partition and retention during cell division. A further role for EBNA-1 could be to prevent loss of newly synthesised plasmids as plasmids containing oriP are efficiently replicated in human cells, but are rapidly destroyed in the absence of EBNA-1 (Aiyar *et al.*, 1998).

Comprehensive understanding of the interactions between EBNA-1 and oriP has allowed the development of expression vectors capable of stable expression of heterologous sequences in mammalian cells. Consequently, vectors based on EBV are now used routinely to generate cell lines expressing novel proteins (reviewed by Margolskee, 1992). The EBV vector is a convenient gene expression system as, after transfection, a much higher number of cells will retain and express an EBV vector

than will stably integrate a non-replicating vector (Sclimenti and Calos, 1998). Multiple copies of the EBV vector may be present per cell, leading to higher expression levels of heterologous sequences. This, together with predictable gene expression that is free from position effect suppression that often affects randomly integrated DNA, makes establishing stable cell lines a more straightforward task.

3.4. The adenovirus major late promoter (MLP).

The adenovirus major late promoter (MLP) controls expression of the major late transcription unit (MLTU) that encodes most of the structural proteins and several non-structural proteins. The MLTU extends from the promoter at 16.8 map units to a termination signal close to the right hand end of the genome at 91.3 map units. mRNAs originating from the MLTU are grouped into five families (designated L1 to L5), where each family consists of mRNAs that have co-terminal 3' ends. These are produced by alternative poly (A) site utilisation and splicing (discussed in section 1.3.5.4.1).

The MLP of subgroup C adenoviruses is one of the most intensively studied examples of a eukaryotic RNA polymerase II promoter. Early studies, using *in vitro* transcription and plasmid-borne transfection assays, defined the requirements for *cis*-acting sequences for quantitative and accurate transcription initiation (reviewed by Berk, 1986). These early studies identified several transcriptional elements and two cellular transcriptional activating factors. More recently, genetic systems were developed to examine the function of the MLP in the context of the viral genome (Logan and Shenk, 1986; Brunet *et al.*, 1987; Reach *et al.*; 1990). Together, these results strongly support the promoter structure shown in figure 3.1. The MLP contains two basal elements, a TATA box that is the binding site for the cellular transcription factor TFIID (Sawadogo and Roeder, 1985, Nakajima *et al.*, 1988) and an initiator element (INR) centred on the start site (Concino *et al.*, 1984). The MLP also contains two upstream activating elements, the upstream promoter element (UPE) that binds the cellular upstream stimulatory factor (USF) (Sawadogo and Roeder, 1985; Gregor *et al.*, 1990) and an inverted CAAT box that binds the cellular transcription factor CP1 (Chodosh *et al.*, 1988, Maity *et al.*, 1988). Two further activating elements are located downstream of the transcription start site and are termed DE1 and DE2, which bind DEF-A and DEF-B, respectively (Jansen-Durr *et al.*, 1989; Mondesert *et al.*,

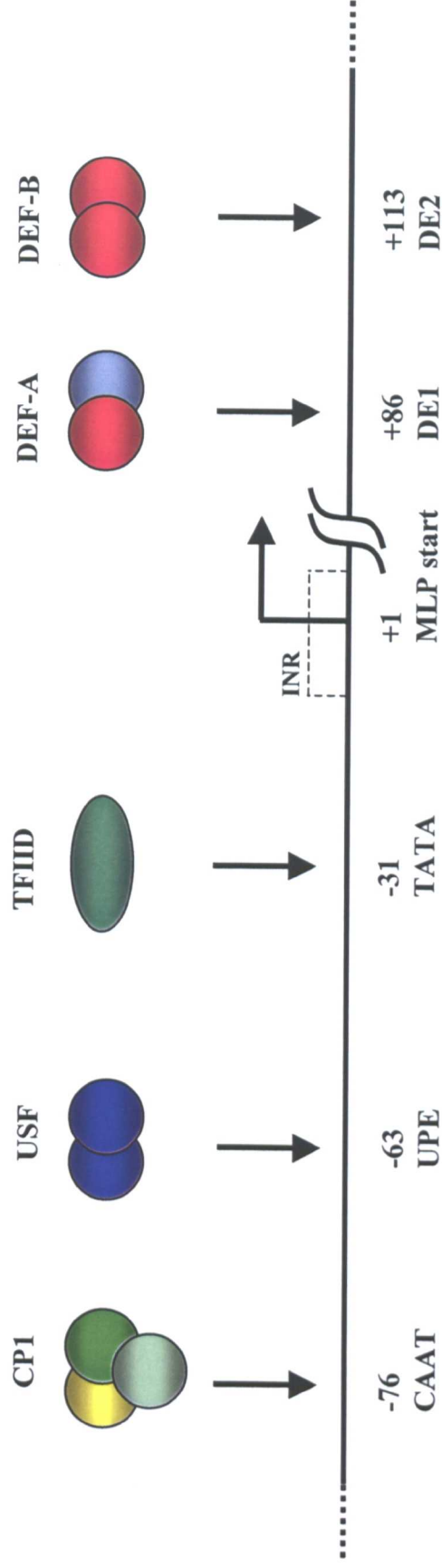


Figure 3.1. Genetic organisation of the Ad5 major late promoter. Upstream and downstream elements are shown with their cognate binding factors. The region is located at ~15.5 map units from the left hand end of the viral genome with the MLP start site at bp 6049. Abbreviations: INR, initiator element; DE, downstream element; DEF, downstream element factor; USF, upstream stimulatory factor; UPE, upstream promoter element; MLP, major late promoter.

1992). Recently, DEF-A and DEF-B have been shown to consist of a heterodimer of the virus-encoded IVa2 protein and an unidentified protein, and a homodimer of IVa2, respectively (Tribouley *et al.*, 1994; Lutz and Keding, 1996).

The MLP exhibits a low level of activity early after infection, and it becomes several hundredfold more active on a per molecule basis during the late phase (Shaw and Ziff, 1980). Analysis of the upstream and downstream activating elements has attempted to determine the mechanisms responsible for activation of the MLP. Sequences implicated in the basal activity of the MLP are the TATA box, INR, UPE and CAAT box (Hen *et al.*, 1982, Logan and Shenk, 1986). The TATA box and UPE have been reported to be functionally redundant with respect to the INR and CAAT box, respectively (Reach *et al.*, 1991). Binding of TFIID to the TATA box appears to be the prerequisite for assembly of the initiation complex, which is further stabilised by interactions between TFIID and USF bound to the nearby UPE site (Sawadogo and Roeder, 1985). TFIID also represents, in this promoter, the main target for direct activation by the E1A-13S protein. E1A-13S protein is able to bind to TFIID (Horikoshi *et al.*, 1991) and stimulate transcription from the MLP (Lewis and Manley, 1985; Leong *et al.*, 1988). In addition, the adenovirus DNA binding protein (DBP) has been shown to stimulate MLP activity *in vitro* (Chang and Shenk, 1990), possibly by a mechanism that involves subtle changes in viral DNA structure that enhance USF binding to the UPE sequence (Toth *et al.*, 1992; Zijderveld *et al.*, 1994).

The late-phase-dependent activation of the MLP is specified by sequence elements that have been located downstream from the transcription start site (Mansour *et al.*, 1986; Alonso-Caplen *et al.*, 1988). DNA-binding and transcriptional studies have allowed the delineation of these downstream elements (DE) within the first intron of the MLTU. DE1 is located between +85 and +98; DE2, between +100 and +120. DE1 binds a factor, DEF-A, which also interacts with the distal part of the DE2 site (DE2a), whereas a second factor DEF-B, interacts with the proximal portion of DE2 (DE2b). The binding of these proteins correlates with transcriptional activation of the MLP (Jansen-Durr *et al.*, 1988, 1989; Leong *et al.*, 1990; Mondesert *et al.*, 1992). Purification of DEF-B allowed identification of the protein as the product of the adenovirus IVa2 gene. DNA-binding and transcriptional experiments have confirmed that the IVa2 protein contributes to late phase specific activation of the MLP by binding to DE2 as a dimer (Tribouley *et al.*, 1994). The IVa2 protein has also

been shown to be part of the DEF-A complex (Lutz and Kedinger, 1996), although the identity of the other protein that constitutes the DEF-A heterodimer is still unknown.

In addition to these *trans*-acting factors, a *cis*-acting change in the viral chromosome is also required for activation of the MLP. MLP expression is strongly stimulated after the onset of DNA replication (Shaw and Ziff, 1980; Grass *et al.*, 1987), however, the molecular basis of this replication dependence of the MLP activity is unclear. Using superinfection experiments, Thomas and Mathews (1980) demonstrated that the early-to-late transition is a function of the particular state of the viral DNA template, as induced by its own replication. One possible explanation for this observation is that the DNA replication process allows transcription factors to gain access to the promoter. Non-replicated, input viral DNA molecules do not appear to be associated with the same set of proteins as replicated viral DNA molecules (Dery *et al.*, 1985; Chatterjee *et al.*, 1986a; 1986b). Therefore, the structure of viral chromatin in the early phase of infection might prevent transcription factors from binding to the MLP, an idea supported by the studies of Toth *et al.* (1992) which showed that DNA replication facilitates the binding of USF to the UPE sequence. Alternatively, transcription factors may not bind to pre-formed viral chromatin, instead they might gain access to the viral template during the process of DNA replication as nucleosomes are disassembled and reassembled. This idea is consistent with earlier work showing that USF cannot activate a promoter that has been pre-assembled in chromatin while it can compete for template binding during the assembly process and subsequently activate transcription (Workman *et al.*, 1990).

3.5.0.(Supplemental). Strategy for the construction of an episomal expression plasmid containing the Ad5 MLTU.

The aims of the strategy were to clone the MLTU into a pBR322-derived plasmid, with *PacI* sites flanking the construct, to enable more straightforward manipulations of the MLTU to be performed during any further studies. This MLTU sequence would then be excised via its *PacI* sites and cloned into an episomal expression plasmid derived from pMEP4 (Invitrogen). This plasmid would be modified prior to use by removal of the human metallothionein promoter (phMTIIa) and the introduction of a unique *PacI* site, into which the MLTU construct would be inserted. Thus the MLTU would remain under the control of its own promoter, rather

than any exogenous vector-derived promoter. The overall cloning strategy is outlined in figures 3.2.suppl.A and 3.2.suppl.B.

3.5. Construction of plasmid pAdJPacI.

Plasmid pFGdXI (figure A.1, kindly donated by Prof. F.L. Graham, McMaster University, Canada), a plasmid containing Ad5 bp 21562 – 35938, with a deletion in the E3 region, was digested with *Bam*HI and *Spe*I. The resulting 6978 bp fragment was cloned into *Bam*HI / *Spe*I cut pBR322*Spe*I (figure A.2) to generate plasmid pAdJYI. Plasmid pAdJYI contains Ad5 bp 27082 – 35938, with a 1878 bp deletion between the two *Xba*I sites at 28592 and 30470. Plasmids bearing the correct insert were determined by restriction enzyme analysis using *Eco*RV (figure 3.2), the bands of 2696 and 2374 bp show that the insert is present and in the correct orientation.

3.5.1. Insertion of unique *Pac*I restriction site.

The target position for insertion of the *Pac*I restriction site relative to the viral transcription unit is shown in figure 3.3.

Two pairs of primers were used to amplify two overlapping regions of the Ad5 genome, using plasmid pAdJYI as the template. Primer sequences are given in section 2.1.6. PCR reactions were performed as described in section 2.3.1.12. using Vent[®]DNA polymerase. Primer #1 (anneals to Ad5 bp 30994 – 31013) and Primer #3 (anneals to Ad5 bp 32862 – 32884 and has a 5' extension GATCTGTTAATTAA, which contains a *Pac*I restriction site) were used to amplify a 1903 bp product (figure 3.4A). Primer #2 (anneals to Ad5 bp 33305 – 33324) and Primer #4 (anneals to Ad5 bp 32890 – 32909 and has a 5' extension CATAGCTTAATTAA containing a *Pac*I restriction site) were used to amplify a 448 bp product (figure 3.4A). Generation of these two products was monitored by agarose gel electrophoresis (figure 3.4B). Optimum amplification of Products A and B was achieved with an MgSO₄ concentration of 2 mM (figure 3.4B, lanes 2 and 5).

PCR product of 2338 bp (spanning Ad5 bp 30994 – 33324, with a *Pac*I site at 32884) was produced by combining products A and B at a 1:1 molar ratio and heat denaturing them at 90 °C for 1 minute. They were then cooled slowly to 25 °C to

Figure 3.2.suppl.A. Overall cloning strategy. Part A.

Step 1: In order to insert a unique *PacI* site at the right-end of the MLTU, the right-end of the Ad5 genome (6978 bp *SpeI*-*Bam*HI [linkered right-end] fragment) was sub-cloned from pFGdX1 into pBR322*SpeI* (section 3.5).

Step 2: The above cloning step resulted in the construction of plasmid pAdJYI, a plasmid containing Ad5 sequence from bp 27082- 35938 (figure 3.2).

Step 3: Plasmid pAdJYI was constructed so that a unique *Bss*HII fragment could be removed, engineered by PCR, and then re-introduced. Site-directed mutagenesis was used to insert a unique *PacI* site at bp pos. 32884 (section 3.5.1). This is downstream of the MLTU sequence in a non-coding region. This plasmid was termed pAdJPacI (figure 3.6).

Step 4: Cloning of the full length MLTU was achieved by constructing a plasmid containing the left-end of the MLTU which overlapped with the MLTU sequence in pAdJPacI. Firstly, a *PacI* site was engineered in pBR322 at the *NdeI* site (position 2297). Subsequently, the *Bst*1107I fragment from Ad5 genomic DNA (bp 5764-29010) was ligated into the unique *Bst*1107I site (position 2245) in pBR322*PacI* (section 3.6).

Step 5: The above cloning step resulted in the construction of plasmid pBRZ17I, which contains the left-end of the MLTU with a unique *PacI* site upstream of the MLTU (figure 3.9).

Step 6: The *ClaI*-*SpeI* fragment from pAdJPacI was cloned into *ClaI*-*SpeI* digested pBRZ17I (section 3.7).

Step 7: The above cloning step resulted in the construction of plasmid pAdMLTU which contains Ad5 sequences from bp 5764-35938. Within this sequence is the MLTU flanked by *PacI* sites (figure 3.10).

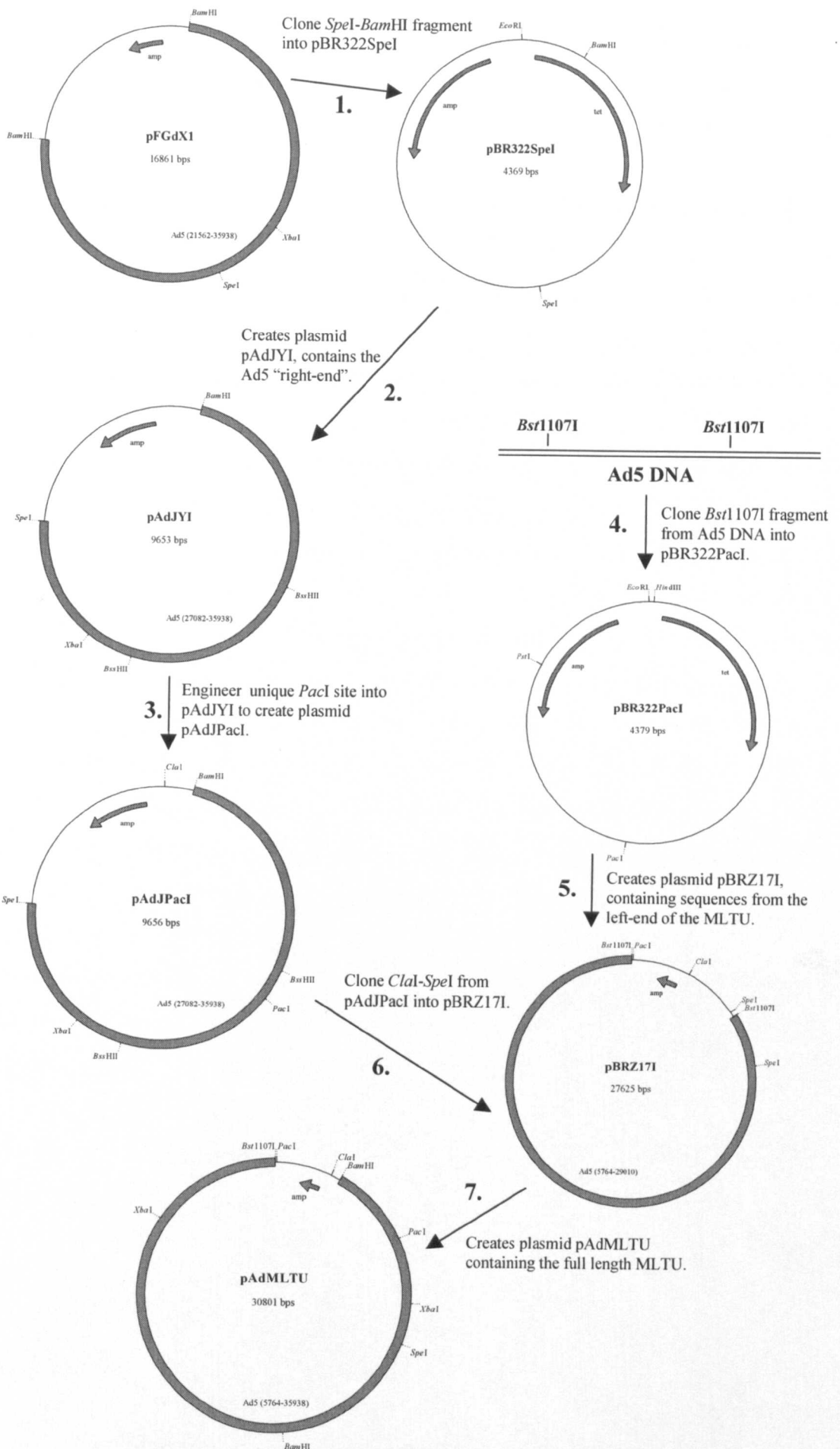


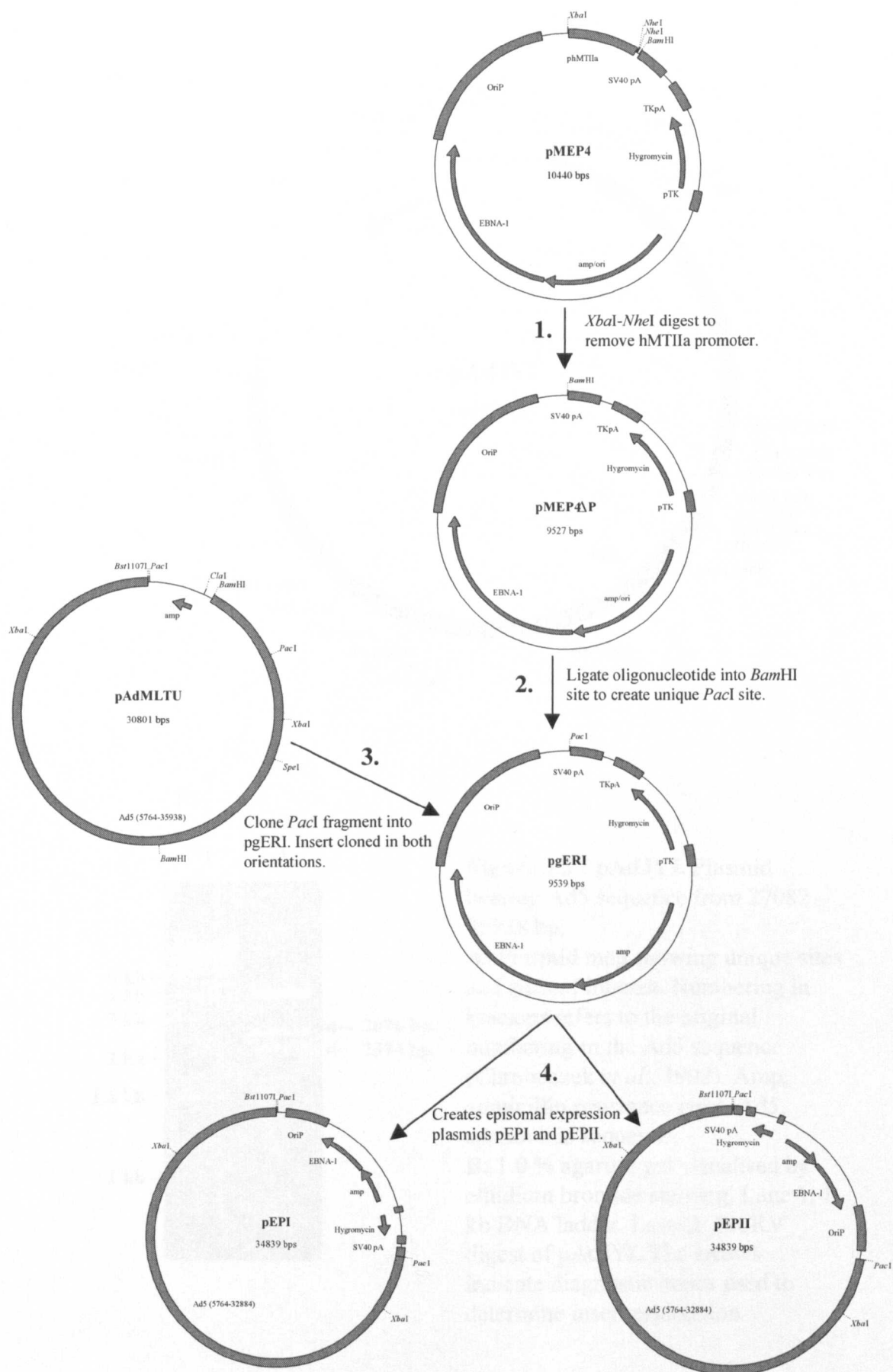
Figure 3.2.suppl.B. Overall cloning strategy. Part B.

Step 1: Expression of the MLTU is driven by the endogenous MLP. The hMTIIa promoter present on plasmid pMEP4 would not be required and was therefore removed by digestion with *Xba*I and *Nhe*I, followed by religation of the linear molecule (section 3.8). This plasmid was termed pMEP4ΔP (figure 3.11).

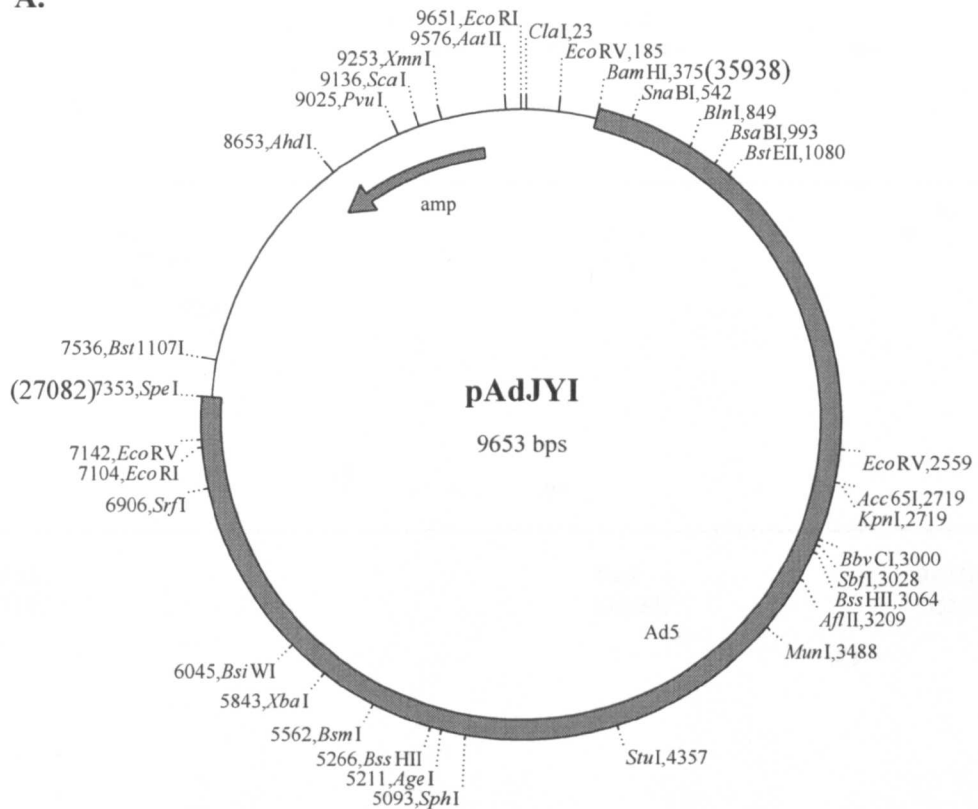
Step 2: An oligonucleotide adaptor was ligated into the unique *Bam*HI site of pMEP4ΔP (section 3.8), thus creating a unique *Pac*I site to facilitate cloning of the *Pac*I-flanked MLTU construct. This plasmid was termed pgERI (figure 3.12).

Step 3: The *Pac*I fragment containing the MLTU was excised from plasmid pAdMLTU and cloned into the unique *Pac*I site present in plasmid pgERI. Clones containing the insert in either orientation were isolated (section 3.8).

Step 4: The above cloning step resulted in the construction of episomal expression plasmids pEPI and pEPII (figure 3.13), which contain the full length Ad5 MLTU and sequences from EBV required for episomal maintenance of the plasmid in eukaryotic cells.



A.



B.

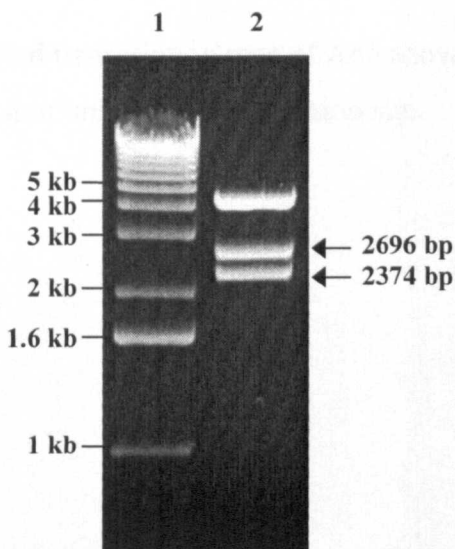


Figure 3.2 : pAdJYI. Plasmid bearing Ad5 sequence from 27082 – 35938 bp.

A: Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp, ampicillin resistance gene. Ad5, adenovirus sequence.

B: 1.0 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb DNA ladder. Lane 2, *EcoRV* digest of pAdJYI. The arrows indicate diagnostic bands used to determine insert orientation.

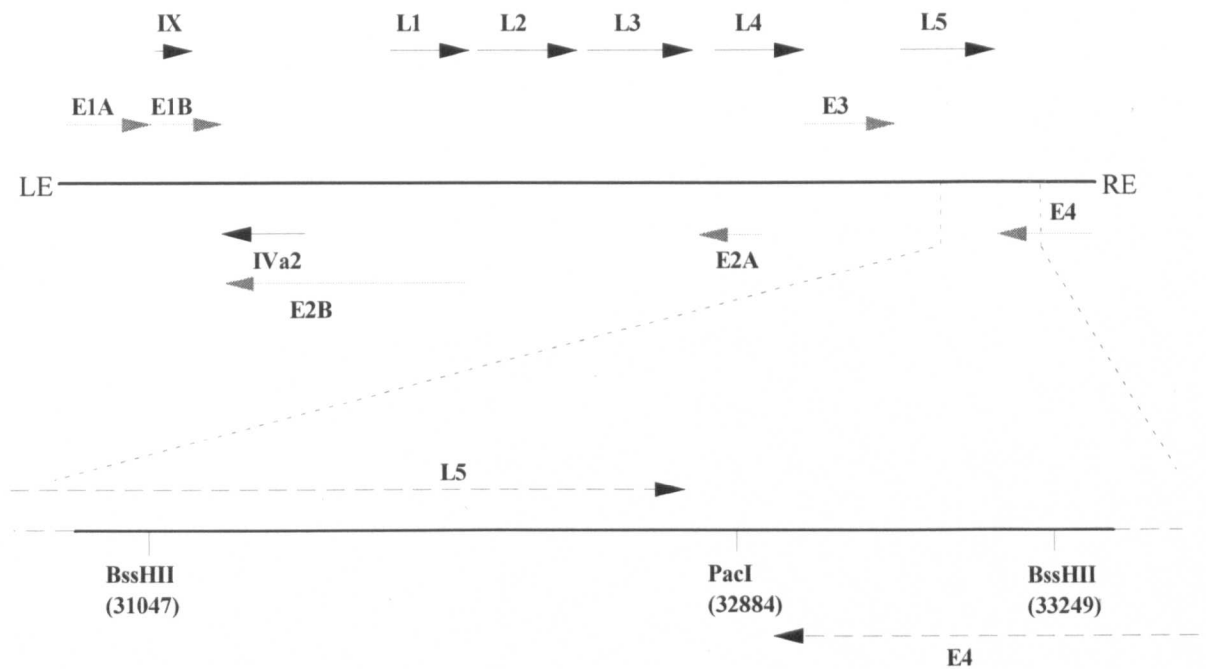


Figure 3.3 : Insertion of a unique *PacI* restriction site by site directed mutagenesis.

Part I.

Simplified transcription map of Ad5 showing the region to be targeted by PCR for insertion of unique *PacI* restriction site.

Figure 3.4 : Insertion of a unique *PacI* restriction site by site directed mutagenesis.
Part II.

A. Two PCR reactions were performed, one using primers #1 and #3 to generate a 1903 bp product (Product A), the other using primers #2 and #4 to generate a 448 bp product (Product B).

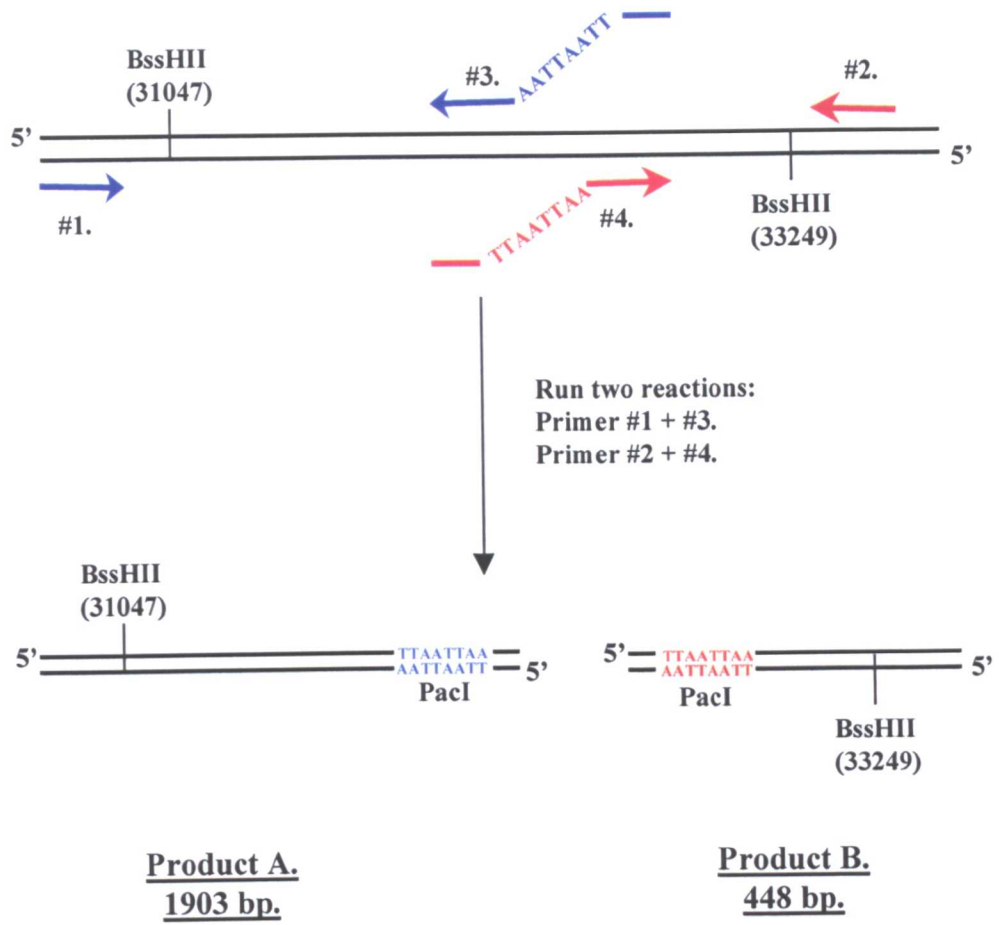
B. 1.2 % agarose gel visualised by ethidium bromide staining.

Lane 1, 1 kb ladder.

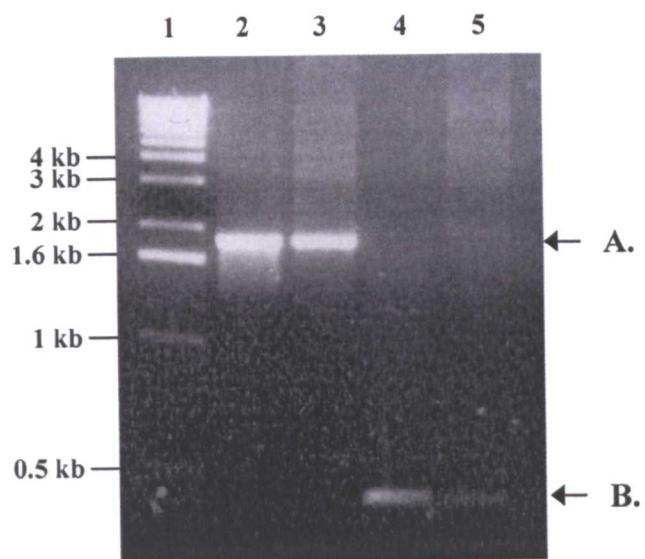
Lanes 2 and 3, Product A from PCR reactions containing 2mM and 4mM MgSO₄ respectively.

Lanes 4 and 5, Product B from PCR reactions containing 2mM and 4mM MgSO₄ respectively.

A.



B.



allow their complementary 3' ends to anneal. Full length template was made by allowing the PCR reaction to proceed for one cycle without primers, followed by addition of Primers #1 and #2 to amplify the full length product (figure 3.5A). Generation of full length product was monitored by agarose gel electrophoresis (figure 3.5B). Production of the full length PCR product was optimum at 4 mM MgSO₄ using a molar ratio of 1:1 (Product A to Product B). These reaction conditions also produced less significant amounts of smaller products when compared to other reaction conditions used.

The full length product was purified by gel electrophoresis / electroelution and cut with *Bss*HII. Plasmid pAdJYI was also cut with *Bss*HII and the PCR product cloned into the dephosphorylated vector, replacing the original Ad5 sequence and creating plasmid pAdJPacI (figure 3.6A). Restriction enzyme digestion with *Bgl*III generated bands of 1672 and 1628 bp (not resolved on gel but diagnosed as a doublet based on intensity) confirming the presence of the PCR insert (figure 3.6B). Digestion with *Eco*RV and *Pac*I (figure 3.6C, lane 4) gave bands of 3714 and 872 bp, showing that the 4586 bp band produced by *Eco*RV digestion alone (figure 3.6C, lane2) had been cut by *Pac*I. This analysis confirmed that the *Pac*I site was present and the insert was in the correct orientation. Automated sequencing (figure 3.7), using primer SEQ-D which anneals to Ad5 bp 32950 – 32969 (section 2.1.6.2), confirmed the presence of the *Pac*I site. This sequence analysis also revealed that a G to C substitution had occurred immediately adjacent to the *Pac*I site. This should not have any effect on expression of L5 reading frames as it is in a non-coding region of the Ad5 genome.

Figure 3.5 : Insertion of a unique *PacI* restriction site by site directed mutagenesis.

Part III.

A. Full length PCR product was produced by heat denaturing Product A and B, then allowing them to re-anneal by their complementary 3' termini. The PCR reaction was allowed to proceed for one cycle before the addition of primers #1 and #2, followed by 19 cycles to generate the 2338 bp product.

B. 1.2 % agarose gel showing production of full length PCR product at varying MgSO_4 concentration and molar ration of Product A : Product B.

Lane 1, 1 kb ladder.

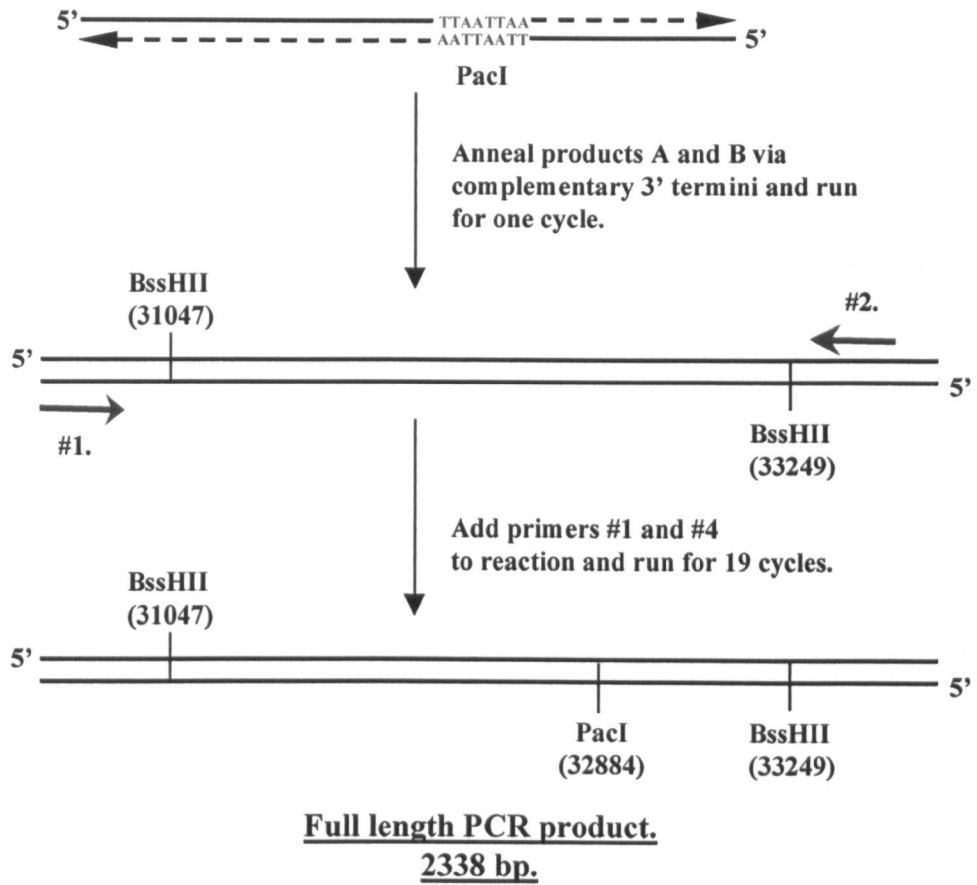
Lane 2, 2mM MgSO_4 , A:B ratio of 1:4.

Lane 3, 2mM MgSO_4 , A:B ratio of 1:1.

Lane 4, 4mM MgSO_4 , A:B ratio of 1:4.

Lane 5, 4mM MgSO_4 , A:B ratio of 1:1.

A.



B.

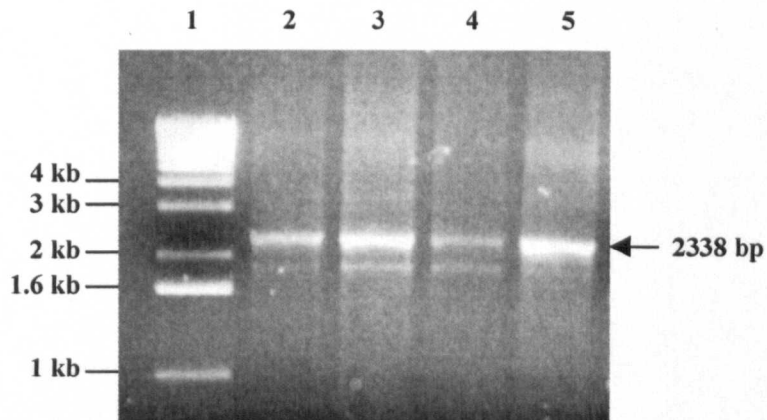


Figure 3.6. Plasmid pAdJPacI.

A. Plasmid map showing unique restriction sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992).

Amp; ampicillin resistance gene. Ad5; adenovirus sequence.

B. 1.4 % agarose gel visualised by ethidium bromide staining.

Lane 1, 1 kb DNA ladder.

Lane 2, *Bgl*III digest of pAdJPacI.

C. 1 % agarose gels visualised by ethidium bromide staining.

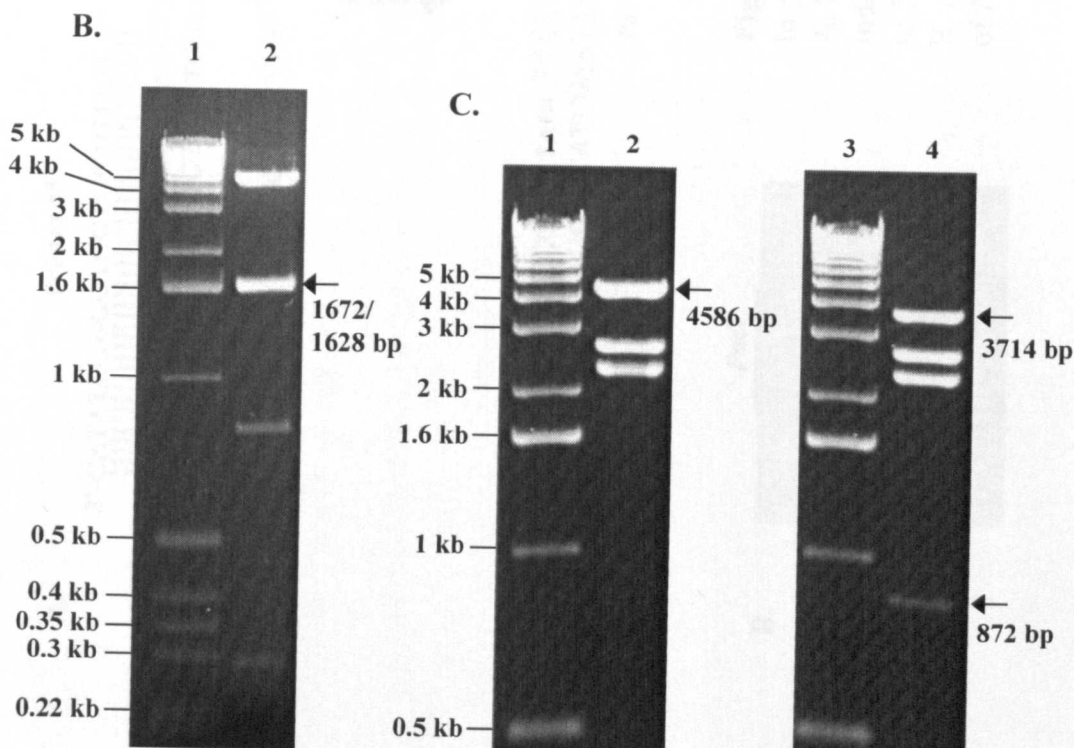
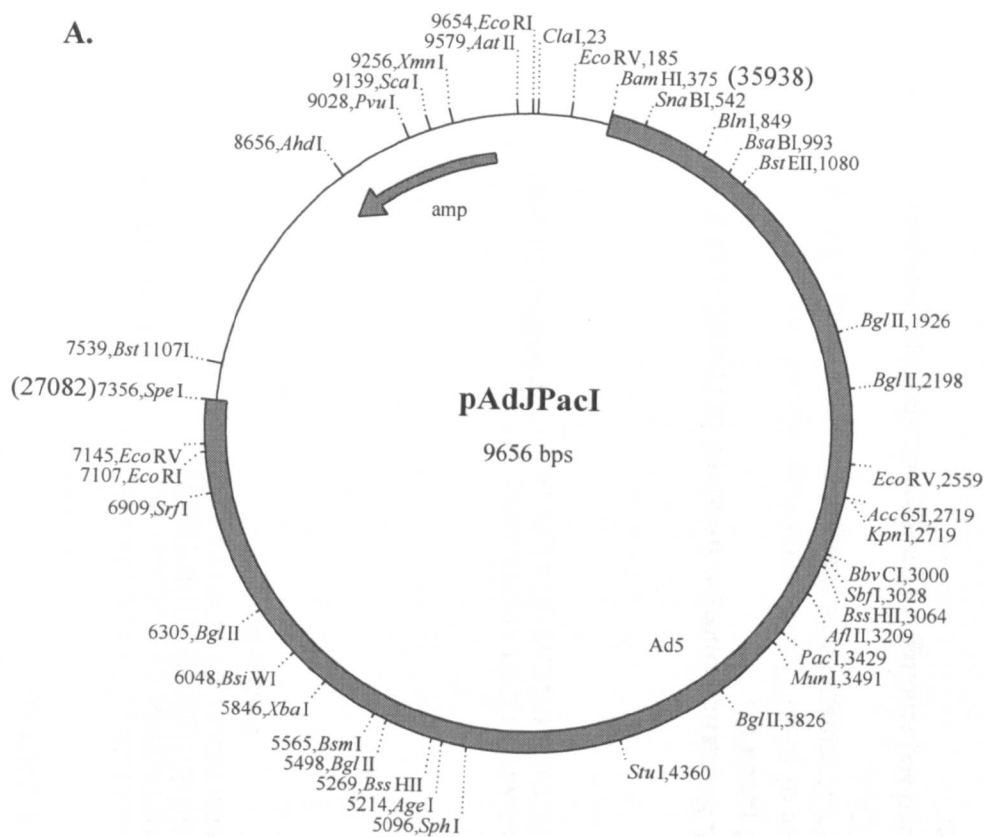
Lane 1, 1 kb DNA ladder.

Lane 2, *Eco*RV digest of pAdJPacI.

Lane 4, 1 kb DNA ladder.

Lane 4, *Eco*RV and PacI double digest of pAdJPacI.

Arrows indicate bands discussed in the main body of text.



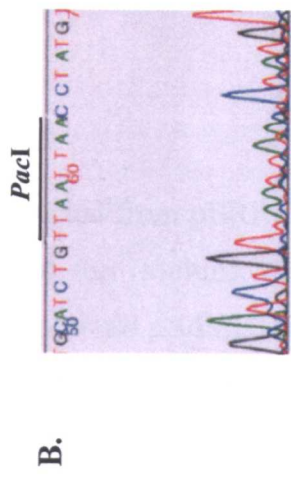
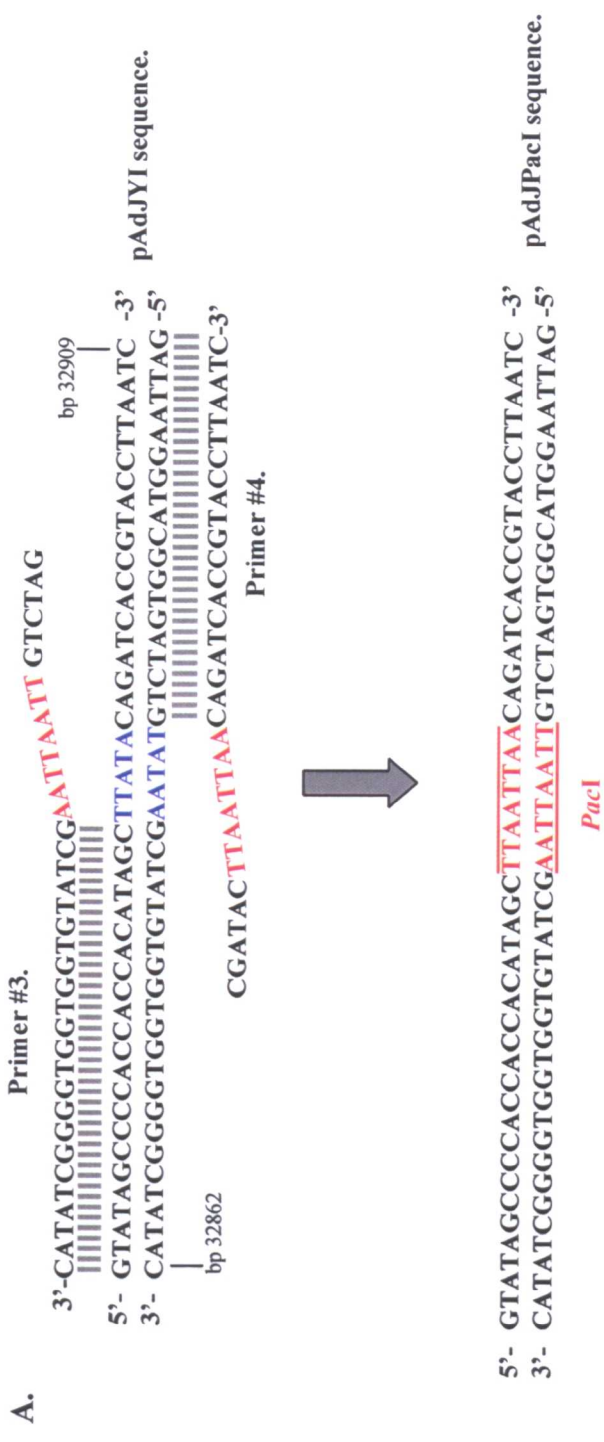


Figure 3.7. Sequence changes involved in insertion of *PacI* site in plasmid pAdJYI.

A. Sequence of pAdJYI with annealing sites of primers indicated. PCR mutagenesis changes sequence from **TTATA** to **TTAATTAA**.

B. Automated sequencing chromatogram showing presence of *PacI* site.

3.6. Construction of plasmid pBRZ17I.

An oligonucleotide adaptor was used to insert a *PacI* site in pBR322 which would ultimately be the *PacI* site at the left end of the MLTU. *NdeI* / *PacI* adaptor (section 2.1.6.3) was heated to 75 °C for 5 minutes and allowed to cool slowly to room temperature, allowing the oligonucleotide to anneal and form a double stranded molecule. Plasmid pBR322 (figure A.3.) was digested with *NdeI* (position 2297) and the adaptor ligated into it to generate plasmid pBR322*PacI*. A positive clone was isolated and presence of the *PacI* site was confirmed by restriction enzyme analysis (figure 3.8). *HindIII*, *PstI*, *PacI* digestion produced bands of 2282 and 1318 bp showing that the *PacI* site was present. Absence of these same bands after digestion with *HindIII*, *PstI* and *NdeI* showed that the *NdeI* site had been removed leaving the 3600 bp fragment uncut.

Genomic DNA isolated from Ad5 was digested with *Bst1107I* and the resulting 23246 bp fragment containing the Ad5 MLP and MLTU segments L1 – L4 was cloned into pBR322*PacI* linearised with *Bst1107I*, creating plasmid pBRZ17I. Plasmids containing the insert in the correct orientation were identified by *EcoRV* digestion (figure 3.9). Diagnostic junction fragments of 5743 and 3784 bp showed that the insert was present and in the correct orientation. Insert in the alternative orientation would show bands at 5498 and 4029 bp. Other *EcoRV* fragments resulting from digestion at sites within the insert (7639, 4546, 2623, 2052, 1238 bp) were all present as expected.

3.7. Construction of plasmid pAdMLTU.

A plasmid containing the full length MLTU was constructed from pBRZ17I and pAdJP*PacI*. pAdJP*PacI* was digested with *ClaI* and *SpeI* and the resulting 7332 bp fragment was cloned into *ClaI* / *SpeI* cut pBRZ17I, creating plasmid pAdMLTU. This plasmid contains the complete Ad5 MLTU flanked by two *PacI* restriction sites. Positive clones were identified by *EcoRV* digestion (figure 3.10, lane 2), bands of 5743 and 2623 bp showed that the insert was present and in the correct orientation. Other fragments of 7639, 4586, 4546, 2623, 2052 and 1238 bp were also present as expected. Digestion with *PacI* (figure 3.10, lane 3) showed the presence of the two *PacI* sites.

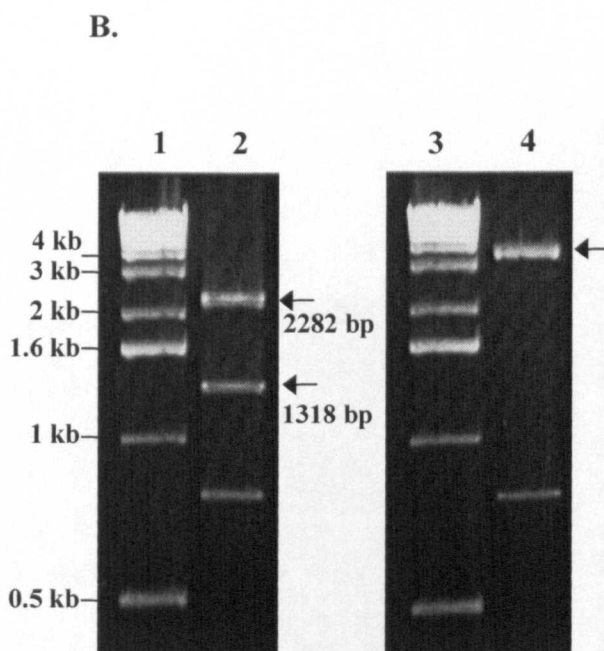
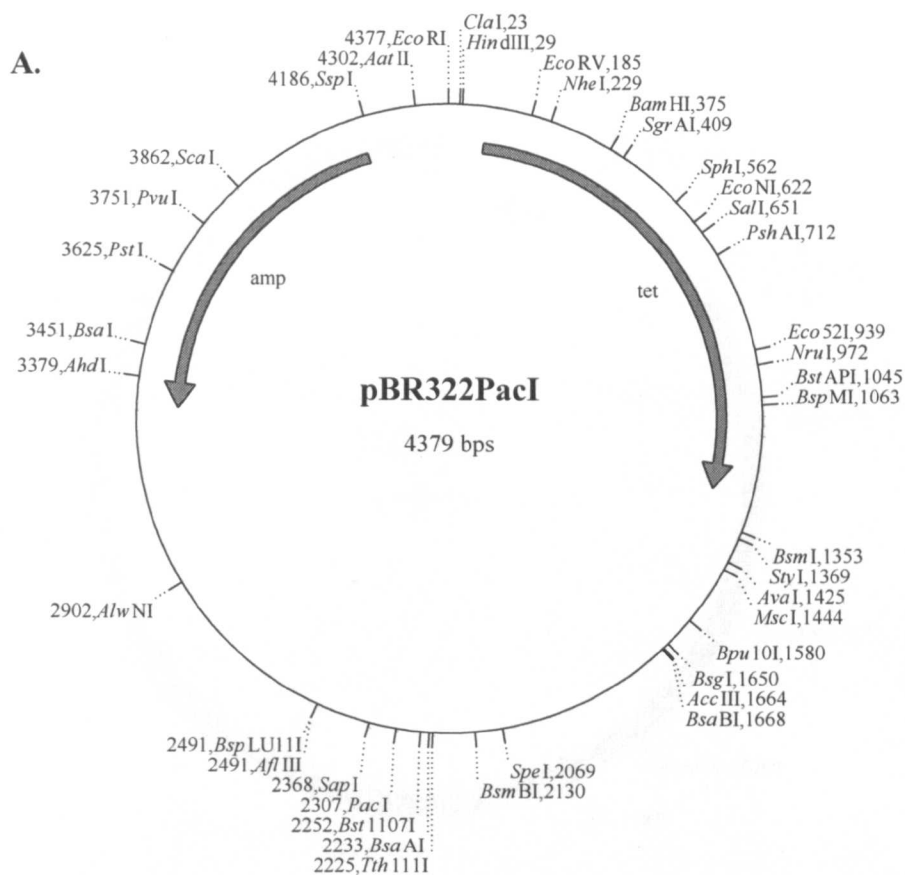


Figure 3.8. Plasmid pBR322PacI.

A. Map showing unique restriction sites. Amp, ampicillin resistance gene; tet, tetracycline resistance gene.

B. 1.2 % agarose gels visualised by ethidium bromide staining. Lanes 1+3, 1kb ladder. Lane 2, HindIII, PacI, PstI digest. Lane 4, HindIII, PacI, NdeI digest. The arrows indicate bands discussed in the text.

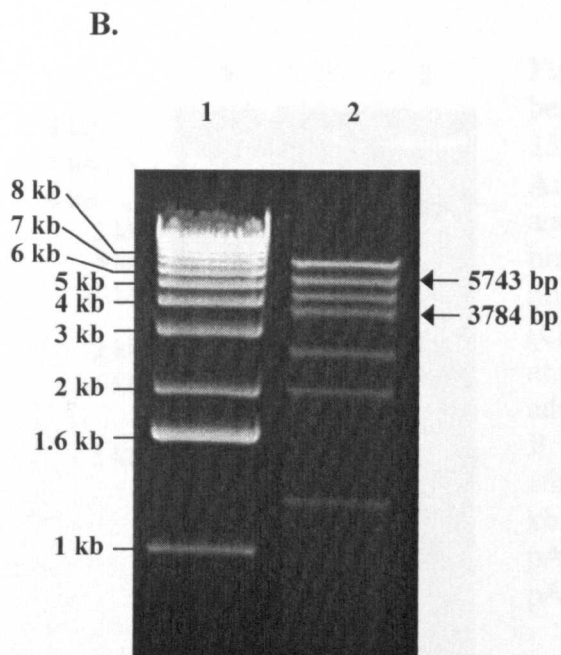
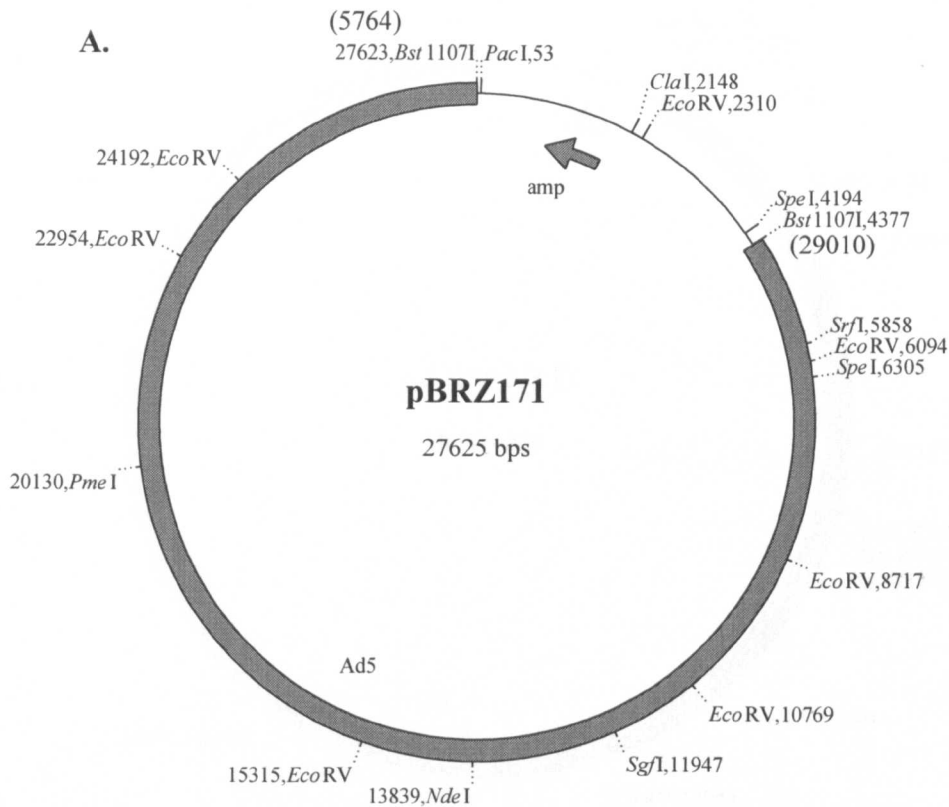
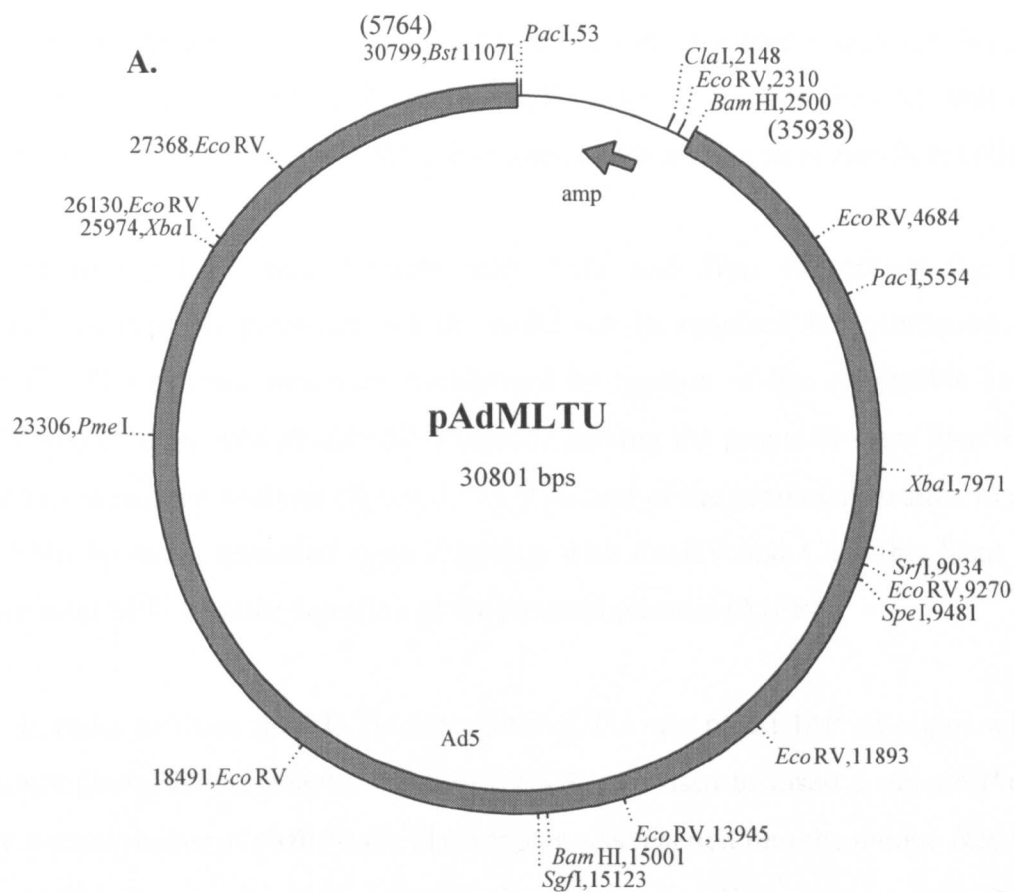


Figure 3.9 : pBRZ171. Plasmid bearing Ad5 sequence from 5764 - 29010 bp.

A: Plasmid map showing unique sites and sites of interest.

Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp; ampicillin resistance gene. Ad5; adenovirus sequence.

B: 1.0 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pBRZ171. The arrows indicate diagnostic bands used to determine insert orientation.



B.

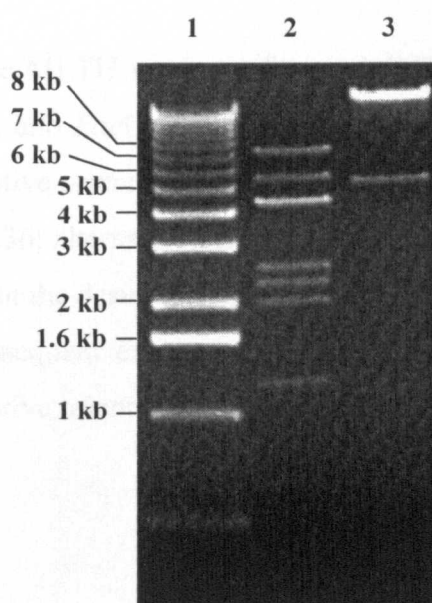


Figure 3.10 . pAdMLTU. Plasmid bearing Ad5 sequence from 5764 – 35938 bp.

A: Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp; ampicillin resistance gene. Ad5; adenovirus sequence.

B: 0.8 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pAdMLTU. Lane 3, *Pac*I digest of pAdMLTU.

3.8. Construction of episomal expression plasmid pEPII.

Plasmid pMEP4 (figure A.4) is an episomal expression vector (previously marketed by Invitrogen) and can be used for transient or stable expression. It contains the EBV sequences oriP and EBNA-1 (discussed in section 3.3) as well as the hygromycin B resistance gene for use as a selectable marker in mammalian cells.

Plasmid pMEP4 was digested with *NheI* and *XbaI* to remove the human metallothionine IIa promoter, which would not be required for expression of the MLTU. The plasmid was then circularised by ligation of the compatible cohesive ends to create plasmid pMEP4ΔP. Plasmids lacking the promoter were identified by restriction enzyme analysis (figure 3.11). Removal of the promoter resulted in a band of 5246 bp being produced upon digestion with *EcoRV* and *ClaI*, this band would have been 6159 bp after digestion of the parental plasmid pMEP4.

In order to clone the MLTU from pAdMLTU into pMEP4ΔP an oligonucleotide adaptor (*Bam*HI-*PacI* adaptor, section 2.1.6.3) was used to insert a unique *PacI* site into the polylinker of pMEP4ΔP. The adaptor was inserted into the unique *Bam*HI site of pMEP4ΔP using the method described in section 3.6. The presence of the *PacI* site was confirmed by restriction enzyme analysis and plasmid pgERI isolated (figure 3.12). A *ClaI* and *PacI* digest produced bands of 4931 and 4608 bp showing that the plasmid was cut by both enzymes and the *PacI* site was present.

The MLTU sequence from pAdMLTU was excised by digestion with *PacI* and cloned into *PacI* cut pgERI. An *EcoRV* digest (diagnostic bands of 12377 and 4364; alternative orientation 12605, 4136 bp) and an *XhoI* digest (diagnostic bands of 9595 and 6236; alternative orientation 15727, 104 bp) were used to identify clones with the insert in the desired orientation (figure 3.13). Plasmid pEPII was used for the majority of subsequent expression analysis. However plasmid pEPI (with the insert in the alternative orientation, figure A.5) was used in the initial expression studies.

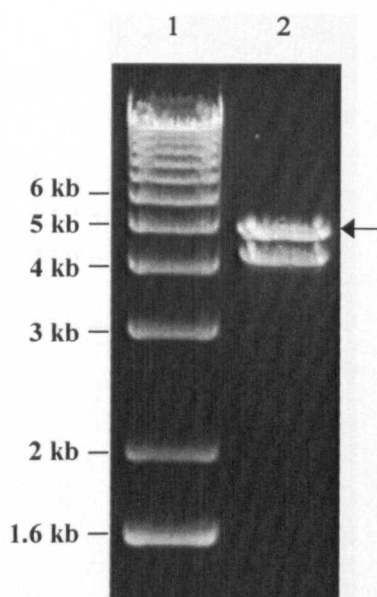
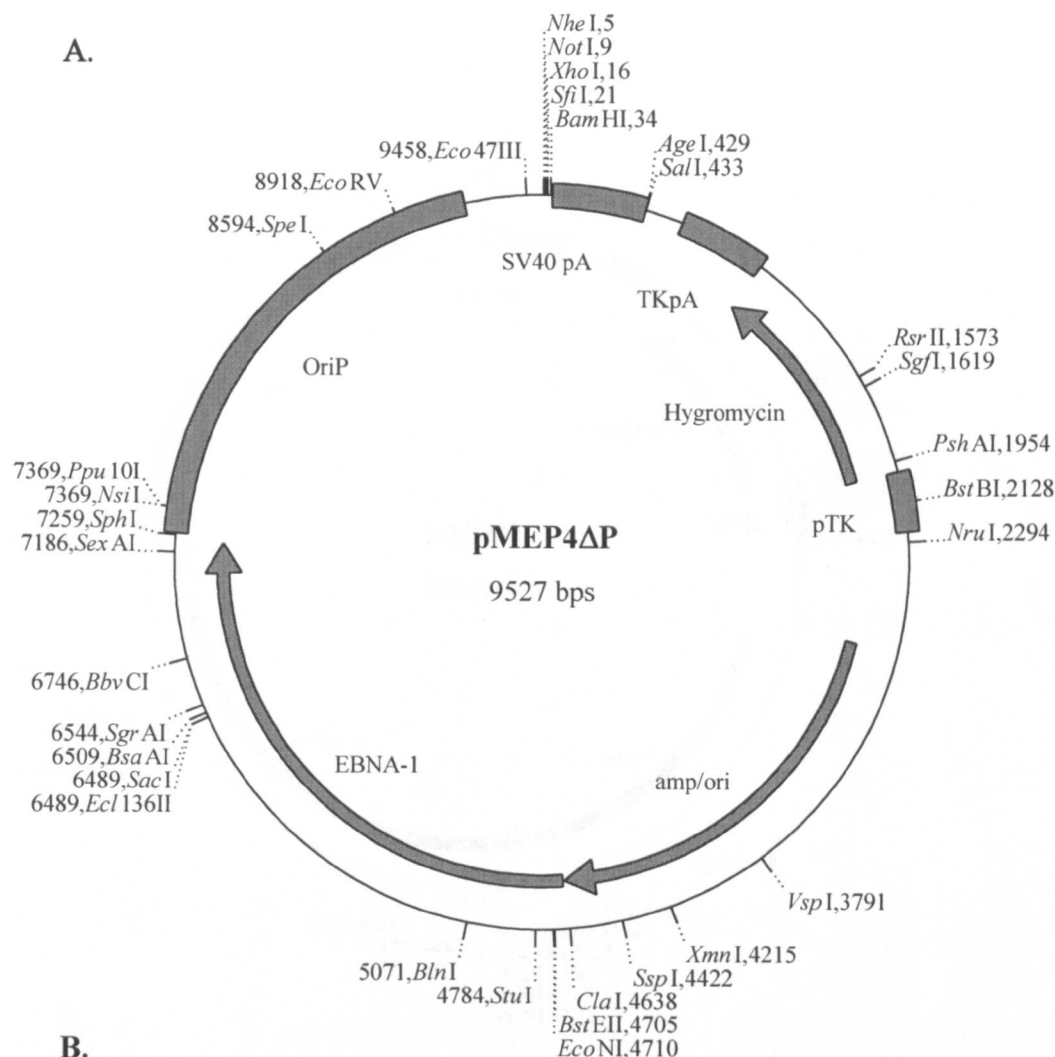


Figure 3.11 : pMEP4ΔP. Derivative of plasmid pMEP4 which does not contain the human metallothioneine IIa promoter (phMTIIa).

A. Plasmid map of pMEP4ΔP showing unique sites.

Abbreviations: pTK, thymidine kinase promoter; Hygromycin, hygromycin-B resistance gene; TKpA, thymidine kinase polyadenylation signal; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp/ori, ampicillin resistance gene/bacterial origin of replication.

B. 0.8 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *EcoRV/ClaI* digest of pMEP4ΔP. The arrow indicates the band discussed in the text.

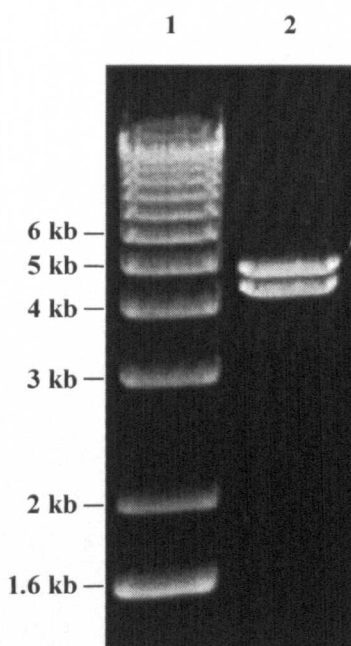
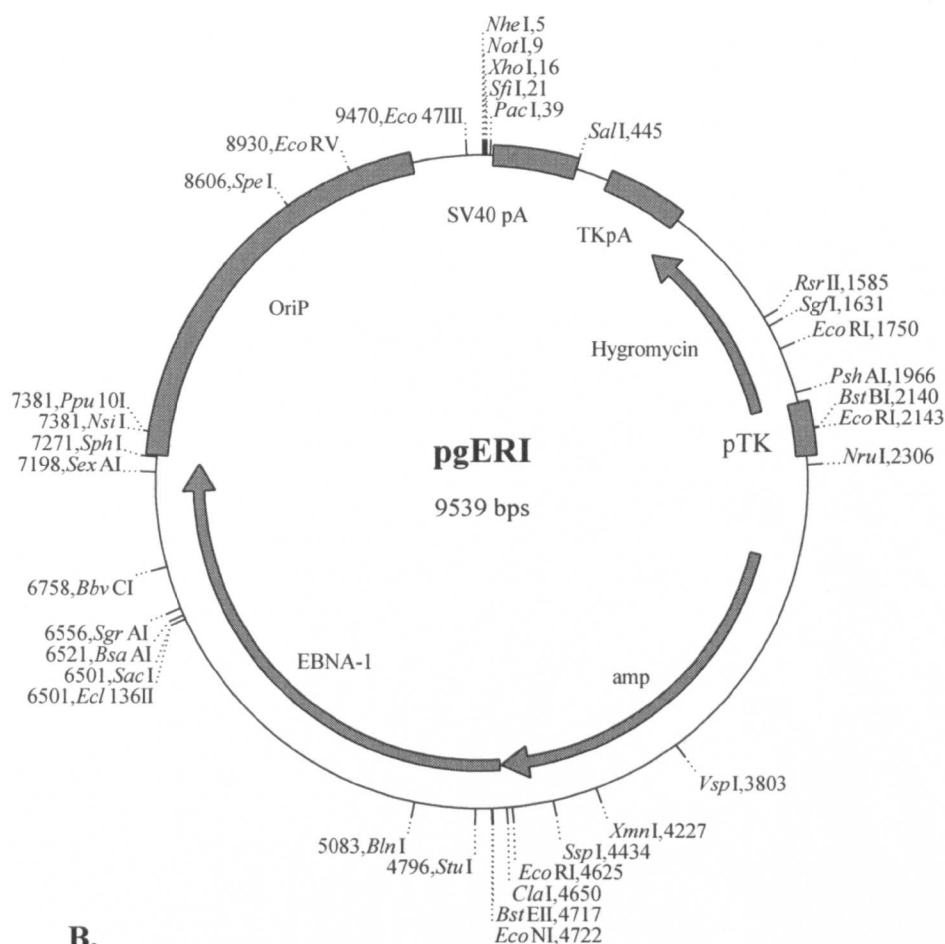


Figure 3.12 : pgERI. Derivative of pMEP4ΔP containing a *PacI* site in the poly-linker.

A. Plasmid map showing unique sites.

Abbreviations: pTK, thymidine kinase promoter; Hygromycin, hygromycin-B resistance gene; TKpA, thymidine kinase polyadenylation signal; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp ampicillin resistance gene/bacterial origin of replication.

B. 0.8 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *ClaI* / *PacI* digest of pgERI.

Figure 3.13 : Plasmid pEPH.

A. Plasmid map showing unique restriction sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.* 1992).

Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1. Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene, Ad5, adenovirus sequence.

B. 0.8 % agarose gels, visualised by ethidium bromide staining, showing restriction enzyme digests of pEPH.

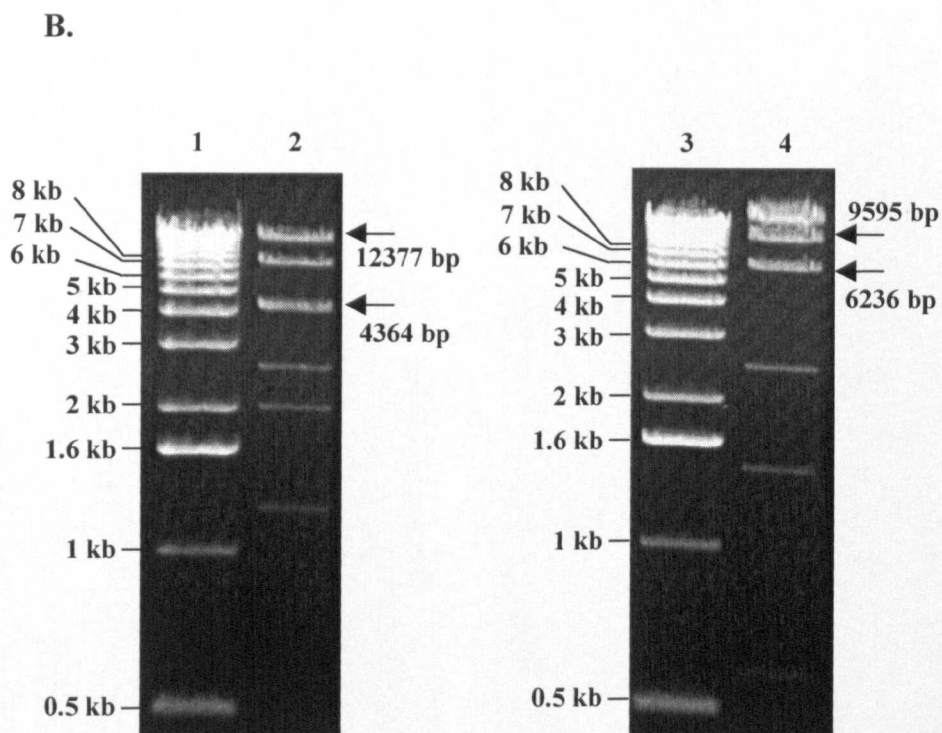
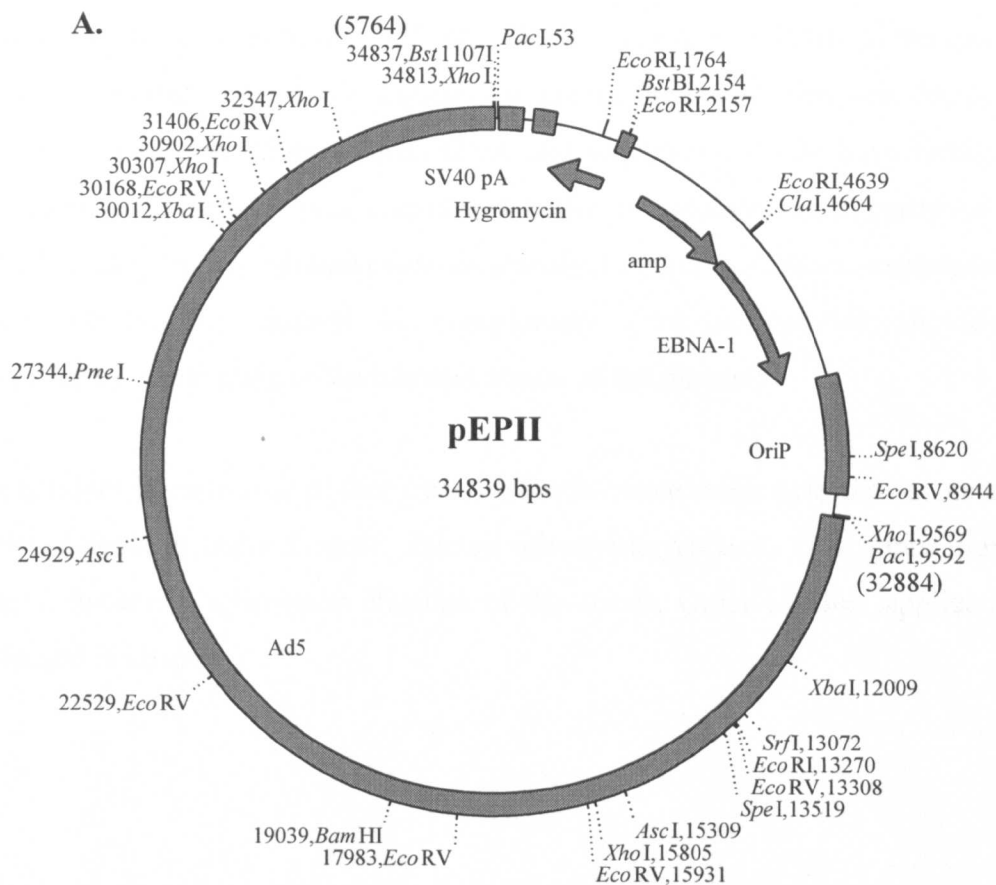
Lanes 1, 1 kb ladder.

Lane 2, *EcoRV* digest of pEPH.

Lane 3, 1 kb ladder.

Lane 4, *XhoI* digest of pEPH.

The arrows indicate diagnostic bands used to determine insert orientation.



3.9. Discussion.

The cloning strategy employed was successful in constructing an episomal expression plasmid containing the Ad5 MLTU. Despite the complexity of the cloning procedure it was decided not to completely sequence the insert. This was due to the fact that there was over 25 kb of Ad5 DNA and sequencing would have been very time consuming. Instead it was considered better to validate the integrity of the individual reading frames through expression analysis. Having achieved expression of specific proteins, any attempt to complement gene deficiencies should be accompanied by sequencing of the relevant region of the plasmid.

The primary intended use of this plasmid was to create stable cell lines expressing Ad5 late proteins in order to grow deleted adenovirus vectors. This application is considered further in subsequent chapters of this thesis. Other possible applications are discussed in chapter 7.

Chapter 4 : Characterisation of late protein expression from plasmid pEP11.

Chapter 4 : Characterisation of late protein expression from plasmid pEPII.

4.1. Introduction.

4.1.1. Proteins encoded by the major late transcription unit (MLTU).

The adenovirus MLTU encodes the capsid and core proteins that are eventually assembled into virions, in addition to several non-structural proteins. A summary of the properties of these proteins is presented in table 4.1. More detailed discussions of their functions can be found in section 1.3.3. (polypeptides II – IX); section 1.3.5.5. (L1 52/55K, L4 100K, viral protease) and section 1.3.5.4.2. (L4 100K).

4.2. Aims.

As already discussed in sections 1.3.5.4, expression of late proteins is regulated at many different levels. One such regulatory role is performed by the major late promoter (MLP), as discussed in detail in section 3.4. Briefly, the promoter shows basal activity in the absence of the viral IVa2 protein, which is a component of MLP-transactivating factors DEF-A and DEF-B. Cellular transcription factor TFIID is involved in basal transcription of the MLP, a process that has been shown to be stimulated by the adenovirus E1A-13S protein. As 293 cells express the E1 proteins, it is conceivable that basal expression from the MLP would occur if the expression plasmid were transfected into 293 cells. Ideally, the MLP would be inactive in 293 cells until stimulated by expressing the IVa2 protein in the same cell. Constitutively high levels of late proteins could cause toxicity problems if a stable cell line were to be isolated which contained the MLTU.

The aim of this work was to determine if any expression of late proteins from plasmid pEPII occurred in 293 cells. If so, what was the extent of this expression. Levels of late protein expression would be determined by Western blot analysis of protein extracts taken from 293 cells transiently transfected with plasmids pEPI or pEPII.

Table 4.1. Products of the adenovirus MLTU.

Gene	Polypeptide	Peptide location / role	Function	Ad2 polypeptide molecular weight (kDa) ^a	Ad5 polypeptide molecular weight (kDa) ^b
L1	52/55K	/	Scaffolding protein involved in virion assembly.	52/55	47
	IIIa	Vertex region	Associated with hexon units, probably serves a bridging function between hexon and VII.	66	65.2
L2	III (penton)	Penton base	Structural protein, forms the penton base.	85	63.3
	V	Minor core protein	Core protein, binds to viral DNA and may form a bridge between core and capsid.	48.5	41.4
	VII	Major core protein	Core protein, probably serves as a histone-like centre around which the viral DNA is wrapped.	18.5	22 ^c
L3	II (hexon)	Hexon	Major structural protein.	120	107.9
	VI	Hexon-associated	Stabilises hexon capsomeres.	24	27 ^c
	23K	/	Thiol protease, cleaves viral-coded precursor proteins required for viral maturation.	23	23.1
L4	VIII	Hexon-associated	Stabilises hexon capsomeres	13	24.7 ^c
	33K	/	Unknown.	33	25.2
	100K	/	Involved in assembly of hexon capsomeres, also required for efficient translation of late mRNAs.	100	90.2
L5	IV (fibre)	Fibre	Recognises cell-surface receptor.	62	61.6

^aThese data are taken from Flint, 1981.

^bPredicted molecular weights based on amino acid composition. Taken from the ExPASy home page (<http://www.expasy.ch>).

^cMolecular weight of the unprocessed precursor polypeptide.

4.3. Preliminary evaluation of expression from plasmids pEPI and pEPII.

A transient transfection assay was used to determine if late proteins were expressed from the plasmids in the absence of any exogenous viral proteins. 10 µg of either pEPI or pEPII (chapter 3) was used to transfect 4×10^6 293 cells in a 60 mm dish using calcium phosphate precipitation. Cells were left for 48 hours before protein extracts were made as described in section 2.3.5.1. For Ad5-infected cell extracts, 4×10^6 293 cells were infected at an m.o.i. of 10 pfu/cell and proteins extracted 24 hours later. Proteins were separated by SDS-PAGE with the protein from 1×10^6 cells loaded per lane for transfections, and from 5×10^4 cells/lane for virus-infected cells. Proteins were then transferred to nitrocellulose membranes and probed with anti-L1 52/55K antibody or a mouse polyclonal antiserum raised against intact wt Ad5 virions (AbBF1) (figure 4.1). The anti-L1 antibody was chosen because L1 52/55K represents an early MLP product (section 1.3.5.4) which might be expected to be expressed even if full transcription of the MLTU and differential splicing of the RNA were not occurring. The anti-L1 52/55K antibody detected a band of the expected size for Ad5 L1 52/55K in extracts from cells transfected with pEPI and pEPII. The band was also present in Ad5 infected cell extracts but not in the mock-transfected cells, thus confirming that the protein expressed from the plasmids was L1 52/55K. Antiserum AbBF1 detected a band of approximately 66 kDa in transfected and infected cell extracts, but which was absent from the mock extracts. Antiserum AbBF1 was raised against intact Ad5 virions and would therefore be expected to have reactivity against the hexon, penton or fibre polypeptides. Based on this and the size of the band, the protein detected was predicted to be the penton polypeptide. The two plasmids expressed approximately equivalent levels of penton polypeptide, with pEPII appearing to express L1 52/55K at a higher level. However, this could be due to variations in gel loading or transfection efficiency. For the remainder of studies only plasmid pEPII was used.

These initial results suggested that at least some expression from the plasmid was possible in the absence of the proposed late promoter activator, the IVa2 protein. However, the plasmids failed to express detectable levels of late proteins when transfected into HeLa cells (data not shown) suggesting that the E1A-13S protein present in 293 cells could be responsible for the observed activation of the MLP. The

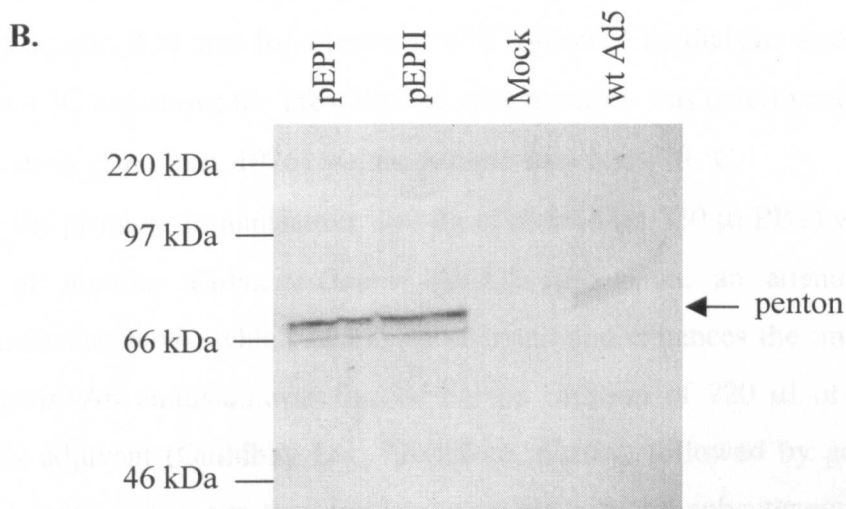
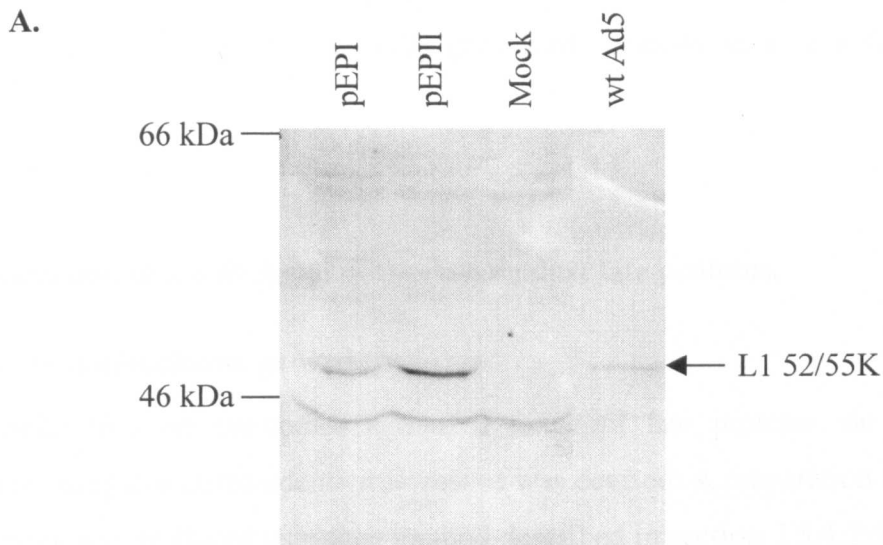


Figure 4.1. Expression of late proteins from plasmids pEPI and pEPII.

Protein extracts from transfected 293 cells, analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with anti-L1 52/55K monospecific antiserum (**A**) and polyclonal antiserum AbBF1 (**B**). The plasmid transfected is indicated above the relevant lane with Ad5 infected-cell extract used as a positive control. Positions of molecular weight markers are indicated on the left, with arrows on the right indicating positions of viral proteins.

antibodies used show expression of proteins from the L1 and L2 genes, with further analysis now required to show expression from other late genes and thus prove that the MLTU primary transcript is being processed correctly to give a full range of mRNAs.

4.3. Production of a polyclonal antiserum against late proteins.

4.3.1. Rabbit inoculation procedure.

In order to raise antibodies against a range of late proteins, an inoculation procedure using denatured adenovirus virions was devised. A preparation of wild type Ad5 virions was produced using the method described in section 2.3.4.2, but omitting the dilution in stabilising buffer. This was then dialysed against PBS for 16 hours at 4 °C, using dialysis tubing with a molecular weight cut-off of 16,000. Virions were then dialysed against 8 M urea for 4 hours at 4 °C, followed by dialysis against PBS for 16 hours at 4 °C to remove the urea. Protein concentration was determined by the Biorad protein assay (Bradford, 1976) and the sample stored at -70 °C.

For the primary immunisation, 100 µg of protein (in 330 µl PBS) was mixed with 50 µl of *Bacillus Calmette-Guerin* (BCG) suspension, an attenuated strain of *Mycobacterium bovis*, which acts as an adjuvant and enhances the immune response to antigens. An emulsion was formed by the addition of 720 µl of non-ulcerative Freund's adjuvant (Guildhay Ltd., Guildford, Surrey) followed by gentle vortexing for 30 minutes. This was then used to inoculate a rabbit subcutaneously. A booster immunisation followed after 4 weeks, when 100 µg of protein (in 380 µl PBS) was again emulsified with 670 µl of non-ulcerative Freund's adjuvant, omitting the BCG. Blood was taken 16 weeks post-immunisation and incubated at 4 °C for 16 hours to allow a clot to form. The clot was removed by centrifugation at 1,300 x g for 10 minutes and the serum frozen at -70 °C.

4.3.2. Analysis of antiserum reactivity.

The antiserum produced from the method described in section 4.3.1, termed AbJLB1, was tested for its ability to react with adenovirus late proteins. Ad5 virions were denatured in 0.1 % SDS and proteins separated by SDS-PAGE and analysed by Western blotting with antiserum AbJLB1 (figure 4.2). The antiserum reacted with several proteins present in Ad5 virions which were also present in Ad5-infected cells, but which were absent from mock-infected cells. An intense band migrating at approximately 120 kDa was predicted to be the hexon polypeptide as this is the major virion constituent and has a molecular weight of 120 kDa in Ad2 virions. Two other proteins of approximately 72 and 60 kDa were predicted to be the penton polypeptide and IIIa respectively, with a further band at approximately 48 kDa most likely to be protein V. A band of approximately 24 kDa present in virion protein samples but not present in infected cells was assumed to be protein VI. This protein is produced by processing of a larger 27 kDa precursor polypeptide (pVI) which can be seen in samples from Ad5-infected cells.

This preliminary evaluation of antiserum reactivity was confirmed by comparison with antiserum AbR1/89 (kindly donated by Dr. V. Mautner, University of Birmingham) that is known to have reactivity against the penton and fibre polypeptides. Comparison of reactivity of AbR1/89 and AbJLB1 with virion proteins (figure 4.3) shows that they both react with the penton polypeptide and that AbJLB1 may also react with L4-100K, which was not previously observed. This data, in conjunction with later expression studies (figure 4.6) shows that AbR1/89 also has reactivity against L4-100K. This is an unexpected observation as both antisera were raised against purified virions and the L4-100K protein has never been detected in mature virions. L4-100K has been shown to act as a scaffolding protein and facilitates the assembly of hexon capsomeres during virus assembly by binding to hexon monomers (Oosterom-Dragon and Ginsberg, 1981; Cepko and Sharp, 1982). Therefore, one possible explanation for the observed L4-100K reactivity is that the virion preparation used for immunisation contained immature virions and therefore also contained L4-100K still associated with the hexon capsomere.

The protein migrating at approximately 60 kDa (figure 4.3, lane 2) has a higher molecular weight than the fibre polypeptide detected by AbR1/89 (figure 4.3, lane 1) and therefore must be IIIa, as this is the only other viral protein in this size range.

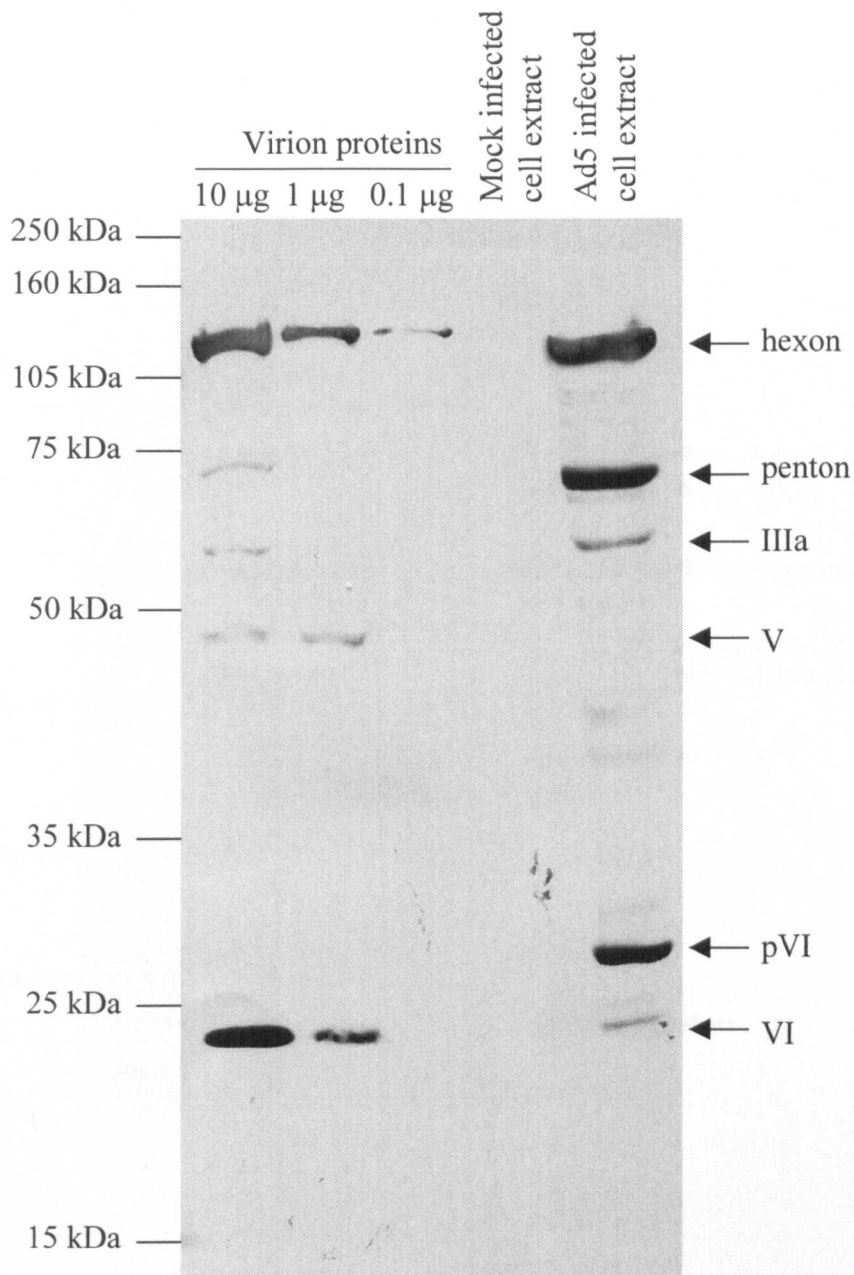


Figure 4.2. Detection of late proteins by polyclonal antiserum.

10 - 0.1 µg samples of denatured Ad5 virions analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with AbJLB1. Also shown are samples from Ad5-infected and mock-infected 293 cells. Positions of molecular weight markers are indicated on the left, with arrows on the right indicating positions of viral proteins.

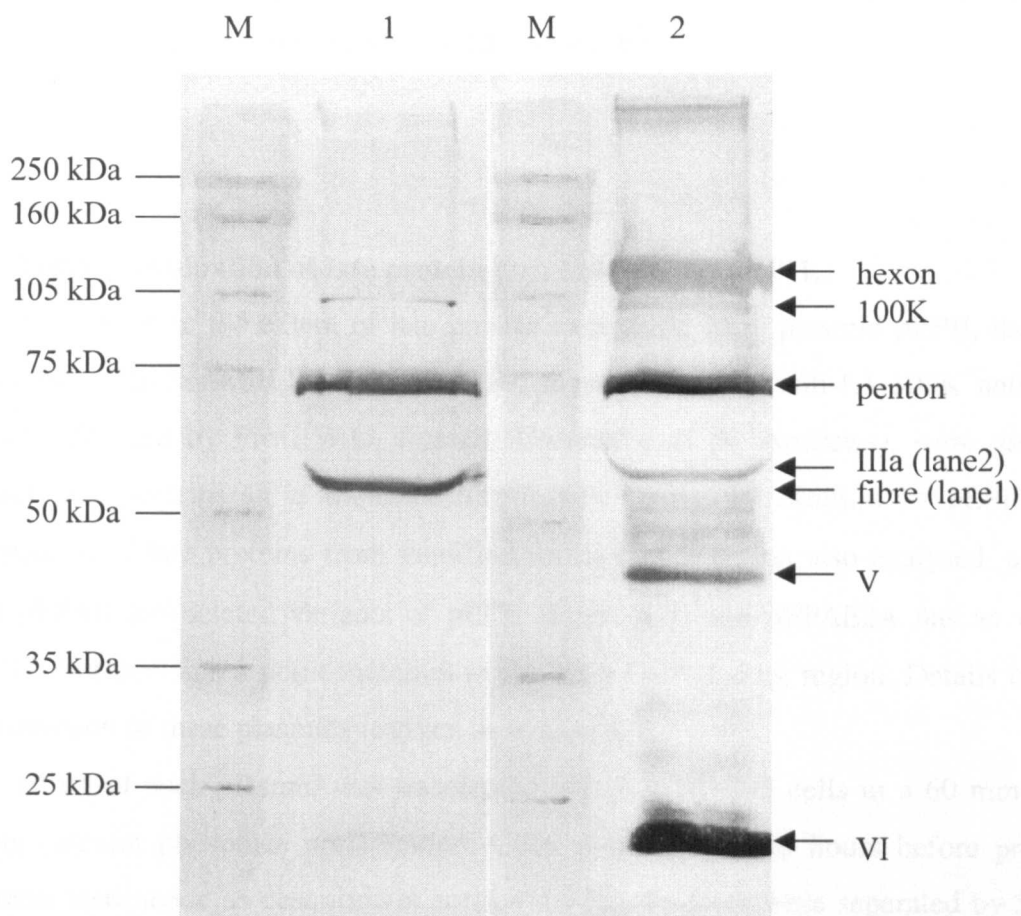


Figure 4.3. Detection of late proteins in adenovirus type 5 virions.

10 μ g samples of denatured virions analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting. Samples were run in duplicate on the same gel, with the nitrocellulose filter subsequently divided and probed with AbR1/89 (Lane 1) or AbJLB1 (Lane 2). Positions of molecular weight markers (M) are indicated on the left, with arrows on the right indicating positions of viral proteins.

Comparison of reactivity of AbR1/89 and AbJLB1 on Ad5-infected cell extracts further supports these observations (figure 4.4). Once again, AbJLB1 reacts with a protein equivalent in molecular weight to the penton polypeptide, and the putative IIIa protein migrates with a higher molecular weight than the fibre polypeptide. Taken as a whole these results suggest that antiserum AbJLB1 has reactivity against a range of late proteins including hexon, penton, IIIa, V and VI.

4.4. Further evaluation of late protein expression from pEPII.

To determine the extent of late protein expression from plasmid pEPII, the two available antisera, AbJLB1 and AbR1/89, together with an anti-L4 100K antibody (kindly donated by Prof. W.C. Russell, University of St. Andrews), were used to detect late proteins in a transient transfection assay. In addition to pEPII, the expression of late proteins from modified forms of pEPII was also analysed. pEPΔI and pEPΔII are deleted variants of pEPII (figure 4.5) and pEPΔE2A has an intact MLTU but contains a point mutation in the E2A-DBP coding region. Details of the construction of these plasmids is given in section 5.4.

10 µg of each plasmid was transfected into 4×10^6 293 cells in a 60 mm dish using calcium phosphate precipitation. Cells were left for 48 hours before protein extracts were made as described in section 2.3.5.1. Proteins were separated by SDS-PAGE with the equivalent of protein from 1×10^6 cells loaded per lane. Proteins were then transferred to nitrocellulose membranes and probed for late protein expression.

Analysis with AbR1/89 reveals the expression of the three proteins known to be detected by the antiserum; penton, fibre and L4-100K (figure 4.6). pEPII expresses all three proteins as does pEPΔE2A. pEPΔI expresses fibre and L4-100K, but not penton as the reading frame has been deleted from the plasmid. There is a faint band migrating at the position predicted for the penton polypeptide, which is also present in pEPΔI samples. This is most likely to be a protein reacting non-specifically with the antiserum, as the band is not as sharp or intense as the band from pEPII and pEPΔE2A samples. pEPΔII expresses the fibre polypeptide, but does not express either penton or L4-100K. A protein of higher molecular weight than 100K is visible (indicated by broken arrow in figure 4.6), which is most likely to be the product of the

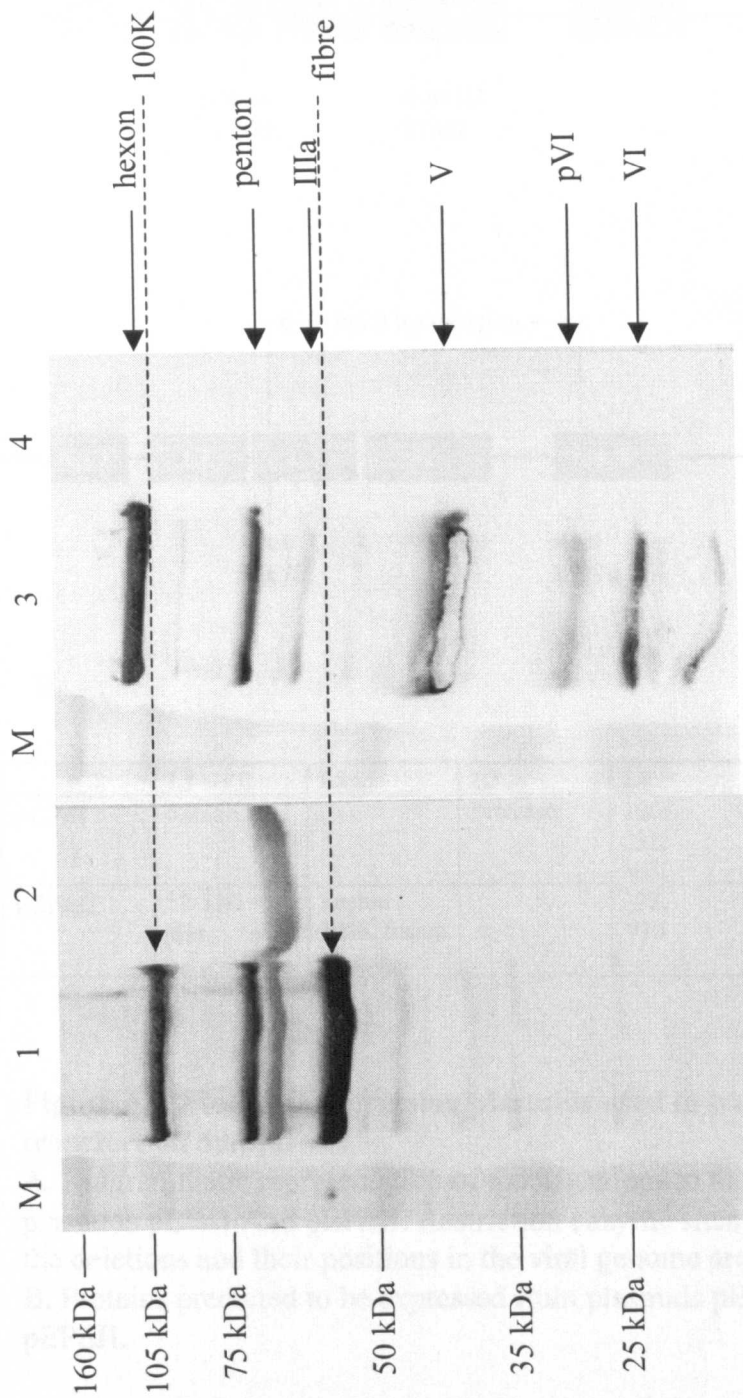
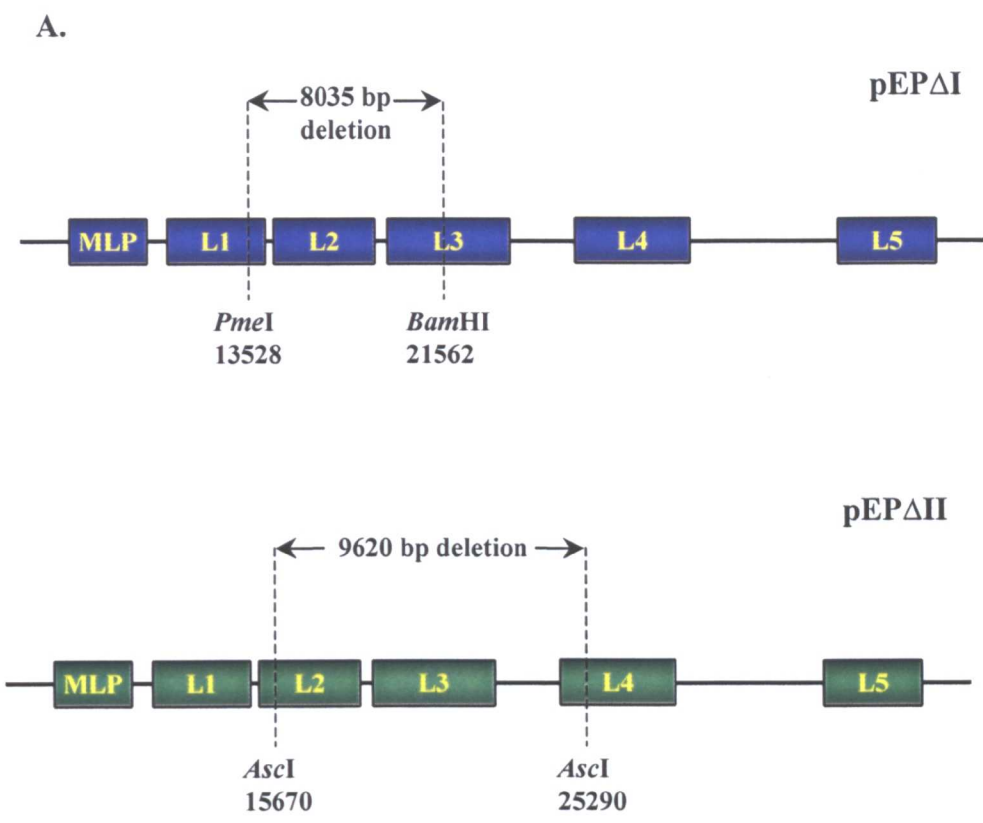


Figure 4.4. Detection of late proteins in adenovirus type 5-infected 293 cells.

Protein extracts from infected (lanes 1 and 3) and mock-infected cells (lanes 2 and 4) analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting. Samples were run in duplicate on the same gel, with the nitrocellulose filter subsequently divided and probed with AbR1/89 (lanes 1 and 2) or AbJLB1 (lanes 3 and 4). Positions of molecular weight markers are indicated on the left, with arrows on the right indicating positions of viral proteins.



B.

	L1	L2	L3	L4	L5
pEPΔI	52/55K		Protease	100K 33K VIII	Fibre
pEPΔII	52/55K IIIa	Penton / 100K fusion protein		33K VIII	Fibre

Figure 4.5. Modified expression plasmids used in transient transfection assays.

A. Diagrammatic representation of modifications to the MLTU of plasmids pEPΔI And pEPΔII. Restriction enzyme sites used to create the deletions and their positions in the viral genome are indicated.

B. Proteins predicted to be expressed from plasmids pEPΔI and pEPΔII.

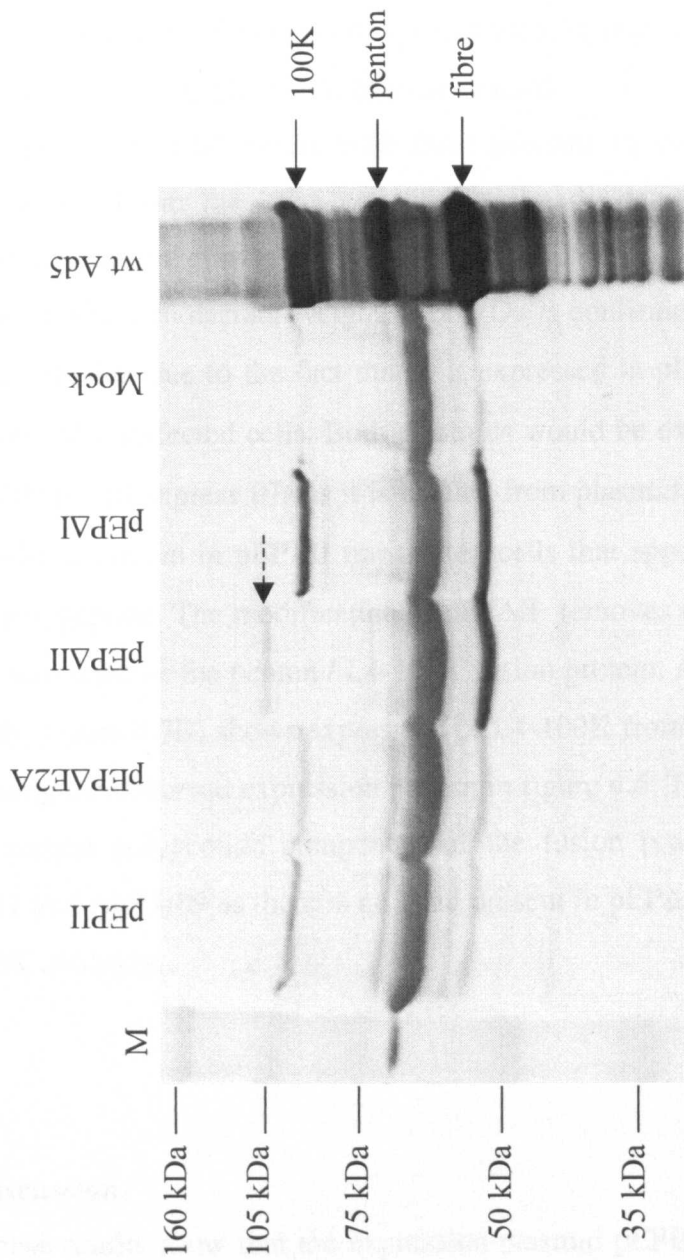


Figure 4.6. Expression of late proteins in a transient transfection assay in 293 cells.

Protein extracts from transfected 293 cells, analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with polyclonal antiserum AbR1/89. The plasmid transfected is indicated above the relevant lane with Ad5 infected-cell extract used as a positive control.

Positions of molecular weight markers are indicated on the left, with arrows on the right indicating positions of viral proteins. The broken arrow indicates the position of the putative penton -100K fusion protein (discussed in section 4.4).

in-frame fusion of the penton and L4-100K reading frames created when the deletion was made in plasmid pEP Δ II. This fusion protein is predicted to have a molecular weight of approximately 100 kDa based on amino acid composition. The deleted plasmids pEP Δ I and pEP Δ II express higher levels of fibre and L4-100K, which is possibly due to there being fewer splicing alternatives available during primary transcript processing. However, this could also be due to variations in transfection efficiency between the plasmid preparations used.

Antiserum AbJLB1 reacts with three proteins in cells transfected with pEP Δ II (figure 4.7A). From the known reactivity already determined for this antiserum, expression of hexon, penton and IIIa from plasmid pEP Δ II is confirmed. The identity of the protein with a molecular weight of 66 kDa is confirmed as being IIIa and not the fibre polypeptide due to the fact that it is expressed in pEP Δ II transfected cells, but not in pEP Δ I transfected cells. Both plasmids would be expected to express fibre but only pEP Δ II will express IIIa as it is deleted from plasmid pEP Δ I. The antiserum also reacts with a protein in pEP Δ II transfected cells that appears to co-migrate with the hexon polypeptide. The modification to pEP Δ II removes the hexon reading frame so this protein must be the penton / L4-100K fusion protein. Analysis with anti L4-100K antibody (figure 4.7B) shows expression of L4-100K from plasmid pEP Δ II and pEP Δ I, confirming the observed expression pattern in figure 4.6. This result also shows that it is the penton polypeptide component of the fusion protein that is recognised by AbJLB1 and AbR1/89 as there is no band present in pEP Δ II samples probed with anti L4-100K antibody.

4.5. Discussion.

These results show that the expression plasmid pEP Δ II is capable of expressing a range of late proteins in transiently transfected cells. The expression of at least one protein from each region of the MLTU has been demonstrated, showing that the full range of poly (A) sites is being used, with a variety of splice sites being selected. This therefore appears to mimic the expression of the MLTU in the context of the viral genome, although it is not possible to say whether the relative levels of expression are identical to those in the late phase of infection.

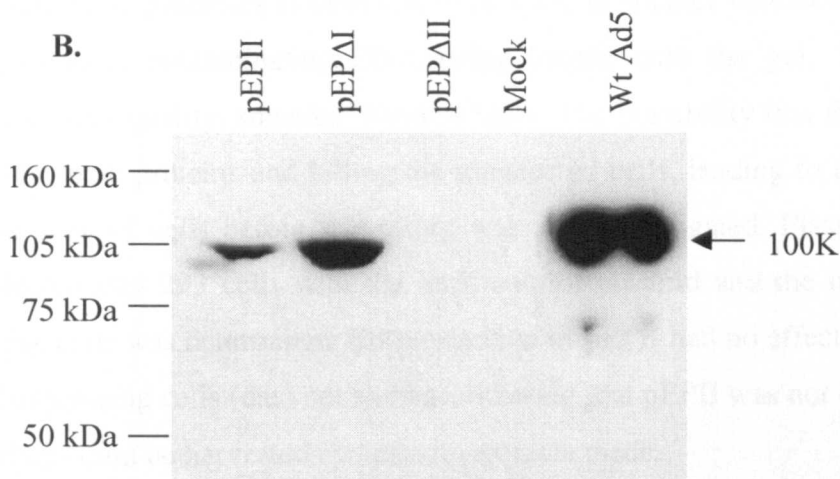
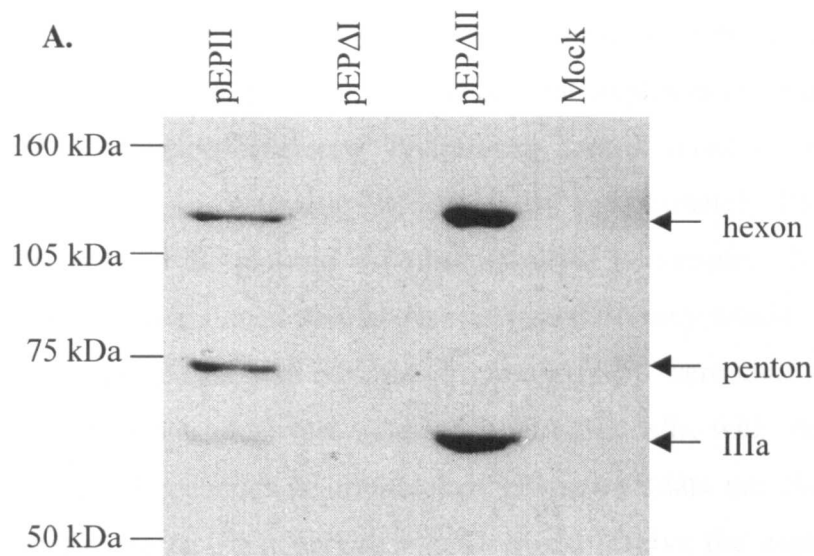


Figure 4.7. Further analysis of late proteins expressed in transfected 293 cells.

Protein extracts from transfected 293 cells, analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with polyclonal antiserum AbJLB1 (**A**) or anti-L4 100K antiserum (**B**). The plasmid transfected is indicated above the relevant lane with Ad5 infected-cell extract used as a positive control. Positions of molecular weight markers are indicated on the left, with arrows on the right indicating positions of viral proteins.

A major problem became apparent in trying to use a transient transfection assay to detect late protein expression. This was that the levels of expression were low and variable. Some transfections failed to show any late protein expression and experiments had to be repeated several times in order to confirm the observed patterns of expression presented in this chapter. One possible explanation for this variability is variation in transfection efficiency. Preliminary control transfections using a *lacZ* reporter plasmid (figure A.6) suggested that only approximately 10 % of cells in a transfection received the plasmid. As plasmid pEPII is considerably larger than the *lacZ* plasmid, it was assumed that the transfection efficiency would be similar, if not lower. To investigate this, two plasmids expressing GFP were constructed, 4 and 30 kbp in size. These plasmids were transfected into 293 cells, with the 30 kb plasmid showing a marked reduction in transfection efficiency (data not shown). To try to compensate for the fact that very few cells would receive the expression plasmid, attempts were made to load more cell equivalents of protein per gel. This in itself caused additional problems as cells had to be lysed in smaller volumes of lysis buffer, leading to more contaminating DNA being loaded onto the gel. This ultimately resulted in poor quality, smeared Western blots. The possibility that the plasmid was expressing toxic proteins and killing the transfected cells, leading to them being lost from the pool of cells before harvesting was also investigated. Plasmid pEPII was cotransfected into 293 cells with the *lacZ* control plasmid and the number of *lacZ* expressing cells was determined. Cotransfection of pEPII had no effect on the number of *lacZ* expressing cells (data not shown), showing that pEPII was not killing the cells before they could be harvested and protein extracts made.

As the expression plasmid pEPII is 37 kbp in size it will be more prone to damage by shearing forces during isolation and subsequent handling. Reducing the number of closed-circular molecules in the plasmid preparation will result in a reduction in transfection efficiency. Steps were taken to minimise damage to the larger expression plasmids, including using the Promega Wizard[®] PureFfection plasmid purification kit, which avoids the use of narrow gauge needles during purification, unlike the CsCl-gradient method. Also, plasmid preparations were stored at 4 °C and not frozen at -20 °C to avoid damage by freeze-thawing.

The levels of expression from pEPII are ultimately difficult to quantify and comparisons with late protein expression in a viral infection are not possible due to

the low number of cells receiving the plasmid. A fair comparison would require stable expression of the late genes from pEPII in a cell line, with the use of viral transactivators to achieve maximum levels of expression from the MLP.

**Chapter 5 : Construction of stable cell lines
containing adenovirus type 5 late protein
expression vectors.**

Chapter 5 : Construction of stable cell lines containing adenovirus type 5 late protein expression vectors.

5.1. Introduction.

Adenovirus vectors offer a wide range of advantages over other viral vectors and therefore represent attractive gene delivery vehicles for gene therapy applications. These advantages include ease of genome manipulation, efficient gene transfer into dividing and non-dividing cells and ease of production of high titre vector stocks. However, the adenovirus vectors currently used in clinical trials have two major disadvantages; the immune response observed upon vector administration and the limited capacity for insertion of heterologous sequences.

Strategies to overcome these obstacles have focussed on the deletion of additional viral coding sequences, in order to reduce the expression of viral antigens and also to increase the transgene carrying capacity of the vector. This approach has been taken to its limit by the production of “gutless” vectors. These vectors are deleted for all viral coding sequences, but retain the ITRs and packaging signal required for replication and packaging of the genome (see section 1.4.5.3). These vectors offer the advantage of not expressing any viral proteins and they can also carry approximately 36 kbp of heterologous DNA. However, they must be propagated in the presence of a helper virus that is difficult to purify away from the vector and can lead to contamination of the vector stock.

Currently available cell lines are not capable of complementing the growth of vectors with all viral coding sequences deleted. Vectors produced in cell lines therefore have lower transgene carrying capacity and will still be capable of expressing a range of viral proteins. Therefore, a cell line capable of supporting the growth of a vector with deletions in multiple gene regions would be beneficial in the development of adenovirus vectors.

The most common approach to the construction of complementing cell lines is to transfect cells with a plasmid containing the desired viral sequences and a selectable marker gene. Integration of the transfected DNA into the cellular chromosome allows transfected cells to be grown in the presence of a selective drug. Expansion of

individual clones allows cell lines constitutively expressing the viral sequences to be isolated. An alternative approach to creating complementing cell lines was used by Caravokyri and Leppard (1995) to construct a cell line expressing pIX. Here expression of pIX was achieved using episomal expression plasmids containing sequences from Epstein-Barr virus (section 3.3). One of the major advantages of this system, compared to the more common method of integrating DNA into the host genome, is that the plasmids are maintained at multiple copies per cell, resulting potentially in a higher yield of expressed protein than from a single integrated construct. Another advantage is that the transfected DNA is maintained episomally, thus preventing mutations that might occur to the cellular genome during the integration process and also avoids any “position effects” on protein expression, i.e. the effect of surrounding chromatin on the expression level.

5.2. Aims.

The aim of this work was to use the expression plasmid pEPII to construct a cell line capable of expressing adenovirus late proteins. This would be achieved by transfecting the plasmid into 293, HeLa and A549 cells, followed by selection of stably transfected cells using hygromycin-B. The extent of late protein expression in any isolated cell line would then be analysed, both with and without the presence of viral transactivating proteins. The ultimate aim of this work being to use the cell line to complement the growth of a highly deleted vector with multiple late gene deficiencies.

5.3. Attempts to construct stable cell lines using pEPI / pEPII.

In order to create stable cell lines a standard transfection and selection protocol was devised. 10 µg of plasmid DNA was used to transfect 4×10^6 cells in a 60 mm dish using calcium phosphate precipitation. At 48 hours post-transfection the cells were passaged 1:3 and after a further 24 hours the growth medium was replaced with DMEM / 10 % NCS containing one of several hygromycin-B concentrations (50 –

Table 5.1. Attempts to construct stable cell lines. 293 and HeLa cells were transfected with a range of plasmids and the isolation of stable cell lines was attempted over a range of hygromycin-B concentrations (50 – 300 µg/ml). Transfections were performed in duplicate and repeated 3 times on separate occasions. + indicates that a stable cell line was isolated. – indicates that a stable cell line was not isolated.

	293 cells					HeLa cells				
	50 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml		50 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	
pEPI	-	-	-	-		-	-	-	-	
pEPII	-	-	-	-		-	-	-	-	
pgERI	+	+	+	+		-	-	-	-	
pCEP-IVa2	+	+	+	+		-	-	-	-	
pBR322	-	-	-	-		-	-	-	-	

300 µg/ml). The growth medium was replaced every 3 days, maintaining hygromycin-B selection, and cells passaged 1:3 when confluent. The results of these initial attempts to isolate stable cell lines are summarised in table 5.1.

This initial test was carried out in 293 and HeLa cells. No cell lines isolated from HeLa cell transfections at any hygromycin-B concentration. The inability of either of the positive control plasmids (pCEP-IVa2 and pgERI) to create cell lines suggested that HeLa cells were either very sensitive to hygromycin-B or that the EBNA-1 protein expressed from all the vectors was toxic to the cells. Transfection of 293 cells resulted in stable cell lines when the control plasmids pCEP-IVa2 and pgERI were used. pCEP-IVa2 was previously used to create a IVa2-expressing 293 cell line (S. Brey, University of Warwick thesis, 1999) and the ability of it to create a stable cell line here showed that the method used was viable. Plasmid pgERI (figure 3.12) contains the EBV replicon but no adenovirus sequences. The ability of this plasmid to create stable cell lines showed that the EBV sequences (oriP and EBNA-1) were functioning correctly. No cell lines could be isolated using the negative control plasmid, pBR322. This shows that it is the EBV sequences present on the other two plasmids that are responsible for their maintenance in stable cell lines.

Despite the control plasmids functioning correctly in 293 cells, neither of the two expression plasmids (pEPI and pEPII) could be used to create a stable cell line. As the vector backbone had been shown to be functional, this inability to be stably maintained in 293 cells must be due in some way to the Ad5 sequences present in the plasmid. Late proteins were shown to be expressed from the MLTU when the plasmid pEPII was transfected into 293 cells (chapter 4). One or more of these proteins may be toxic to the cell, thus preventing any transfected cell from forming a stable cell line.

5.4. Modifications to expression plasmid pEPII.

In an attempt to allow plasmid pEPII to be maintained in 293 cells, strategies were devised to reduce the expression of potentially toxic proteins. These involved two deletions to remove the expression of selected late proteins, and a frameshift mutation was introduced into the E2A DNA binding protein (DBP) reading frame. Although DBP is not expressed from the MLTU, the E2A gene is located within the

MLTU on the opposite strand and may still be expressed from the E2 promoter. DBP is known to be toxic to human cells (Klessig *et al.*, 1984) and has previously been expressed from tightly-regulated inducible promoters to allow the construction of stable cell lines (Brough *et al.*, 1992; Zhou *et al.*, 1996; Zhou and Beaudet, 2000). Therefore, if cells are unable to tolerate constitutive expression of DBP, plasmid pEPII will not be maintained in a stable manner and this would explain the inability to isolate a stable cell line.

The cloning strategy required to introduce a point mutation into the DBP reading frame was complex. Therefore, before undertaking this work, expression studies were performed to determine if the plasmids did express DBP. 10 µg of either pEPI or pEPII (chapter 3) was used to transfect 4×10^6 293 cells in a 60 mm dish using calcium phosphate precipitation. Cells were left for 48 hours before protein extracts were made as described in section 2.3.5.1. For Ad5-infected cell extracts, 4×10^6 293 cells were infected at an m.o.i. of 10 pfu/cell and proteins extracted 24 hours later. Proteins were separated by SDS-PAGE with the protein from 1×10^6 cells loaded per lane for transfections, and from 5×10^4 cells/lane for virus-infected cells. Proteins were then transferred to nitrocellulose membranes and probed with an anti-DBP monospecific antiserum (figure 5.0). Both plasmids pEPI and pEPII were found to express DBP when transfected into 293 cells.

To determine if it was late protein or DBP expression that was causing the toxic effect, the three modified plasmids were constructed and assessed for their ability to create stable cell lines. The modifications and their effects on the number of proteins potentially expressed are summarised in figure 5.1.

5.4.1. Construction of deleted variants of pEPII.

Plasmid pEPII (figure 3.13) was digested with *PmeI* and *BamHI*. The resulting 26531 bp fragment was modified by converting the 5' overhang to a blunt terminus using the Klenow filling method (section 2.3.2.3). The DNA was then circularised to create plasmid pEPAI and positive clones identified by restriction enzyme analysis using *EcoRV* (figure 5.2). Plasmids with the deletion would produce a diagnostic 3881 bp fragment plus fragments of 12377, 4364, 2623, 2052 and 1238 bp; the parent

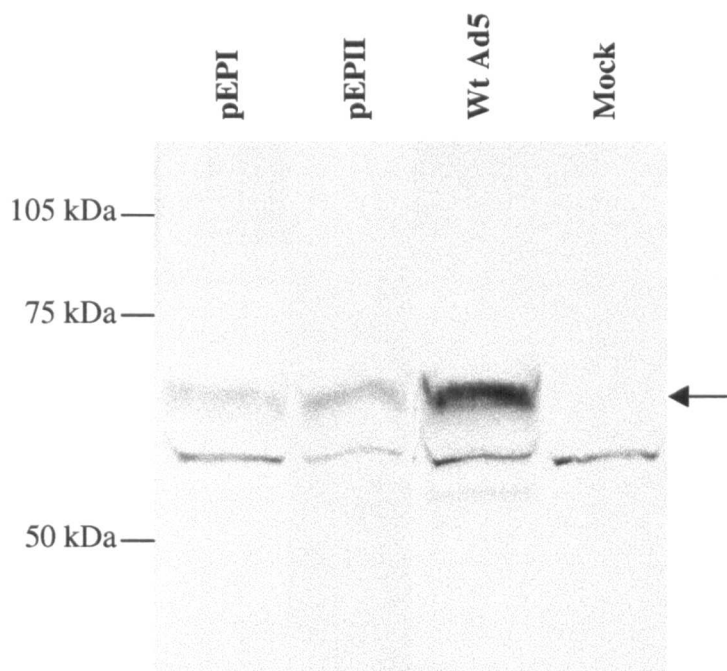


Figure 5.0. Expression of DBP from plasmids pEPI and pEPII.

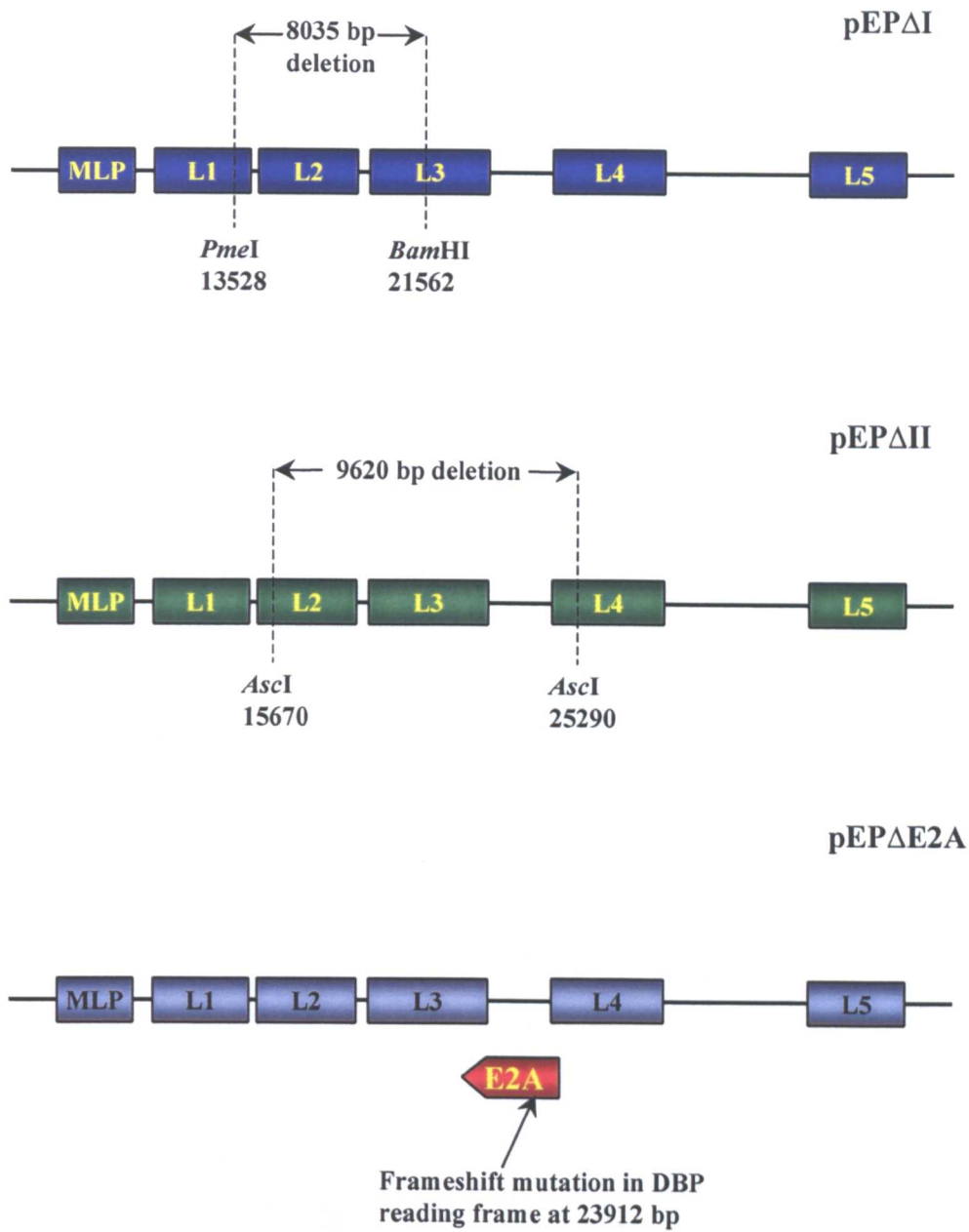
10 μ g of each plasmid was transfected into 4×10^6 293 cells in a 60 mm dish using calcium phosphate precipitation. Cells were left for 48 hours before protein extracts were made as described in section 2.3.5.1. Protein extracts from 1×10^6 cells were analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with anti-DBP antiserum. The plasmid transfected is indicated above the relevant lane with Ad5-infected cell extract used as a positive control. Positions of molecular weight markers (M) are indicated on the left, the arrow on the right indicates the position of DBP.

Figure 5.1. Modifications to expression plasmid pEPII.

A. Diagrammatic representation of modifications made to pEPII to create pEP Δ I, pEP Δ II and pEP Δ E2A. Restriction enzyme sites used to create the deletions and their positions in the viral genome are indicated.

B. Proteins predicted to be expressed from plasmids pEP Δ I and pEP Δ II. Plasmid pEP Δ E2A is predicted to express the same range of late proteins as pEPII.

A.



B.

	L1	L2	L3	L4	L5
pEPΔI	52/55K		Protease	100K 33K VIII	Fibre
pEPΔII	52/55K IIIa	Penton / 100K fusion protein		33K VIII	Fibre

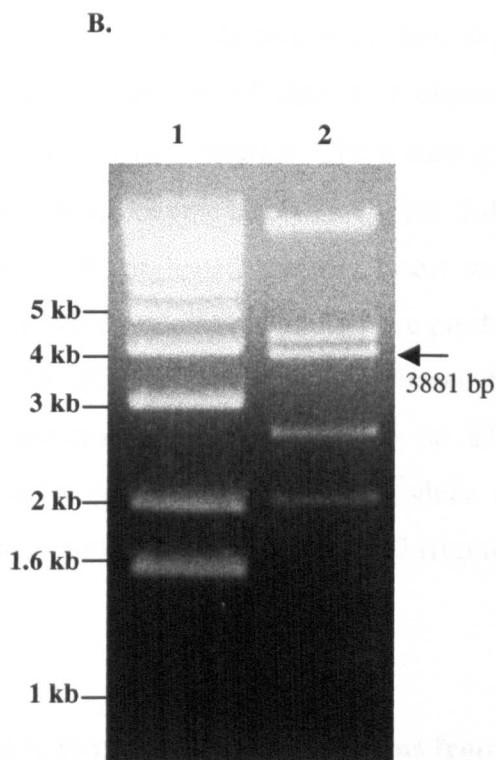
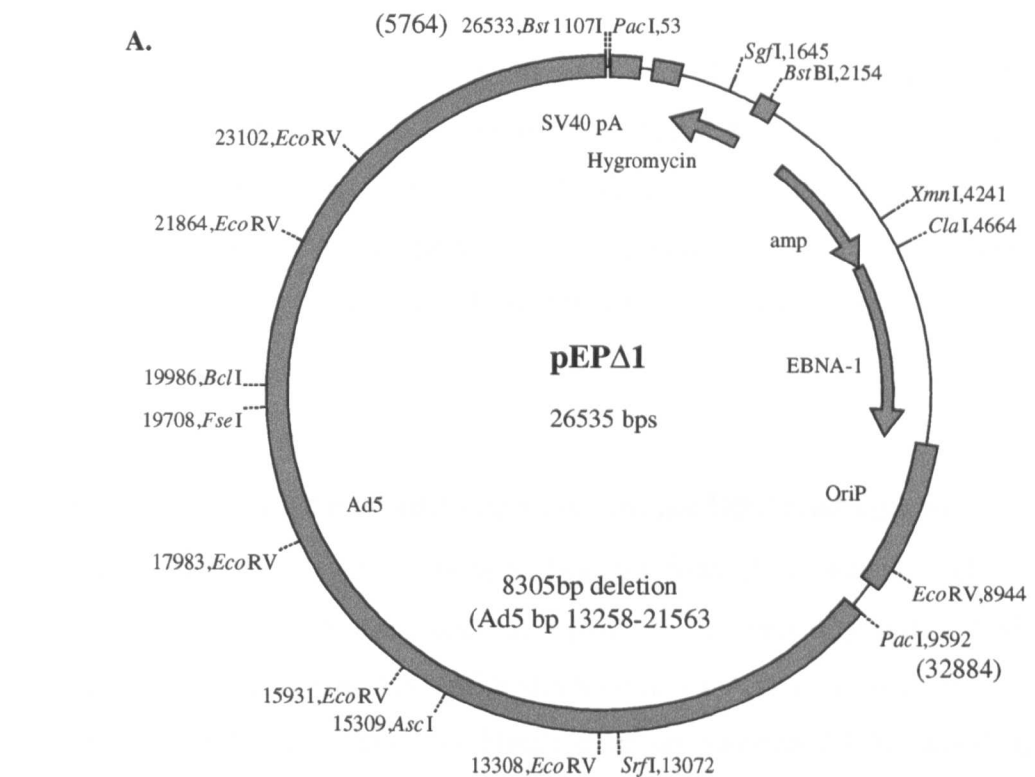


Figure 5.2. Plasmid pEPΔI.

A. Plasmid map showing unique sites and sites of interest.

Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene, Ad5, adenovirus sequence.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pEPΔI. The arrow indicates diagnostic band used to determine that plasmid contains the deletion.

plasmid pEPII would produce 4546 and 7639 bp fragments against this same background.

Plasmid pEPII (figure 3.13) was digested with *AscI*. The resulting 25219 bp fragment was then circularised to create plasmid pEPΔII and positive clones identified by restriction enzyme analysis using *EcoRV* (figure 5.3). The absence of the 4546, 2623 and 2052 bp bands and the presence of the predicted novel junction fragment of 7240 bp upon digestion of pEPΔII with *EcoRV* (figure 3.13) confirmed the deletion.

5.4.2. Introduction of a frameshift mutation into the DBP reading frame.

Plasmid pBRZ17I (figure 3.9) was digested with *PmeI* and *BamHI* and the resulting 8308 bp fragment was cloned into *BstZ17I* / *BamHI* digested pE2(SI)SX (a gift from Dr. Leppard; figure A.8). pE2(SI)SX contains Ad5 sequence from 21562 to 27331 bp, with a *SmaI* to *XhoI* modification at bp position 23912, resulting in a frameshift mutation in the DBP reading frame. This ligation resulted in the generation of plasmid pE2SX-L (figure A.9) which contains Ad5 sequence from 13255 bp to 27331 bp. This plasmid was then digested with *AscI* and the resulting 9620 bp fragment was cloned into *AscI* digested pEPII, replacing the pEPII sequence and introducing the mutation. The resulting plasmid, termed pEPΔE2A, was identified by restriction enzyme analysis (figure 5.4). Bands at 7639 and 2625 bp after *EcoRV* digestion confirmed that the insert was present and in the correct orientation, the alternative orientation would have produced bands of 5859 and 4403 bp. The presence of the DBP mutation was confirmed by *XhoI* digestion (figure 5.4). The mutation introduces an *XhoI* site at Ad5 bp 23912, upon digestion with *XhoI* any plasmid containing the mutation will produce an extra band of 886 bp (figure 5.4, lane 4) when compared to plasmid pEPII (figure 5.4, lane 6).

5.4.3. Expression of late proteins from modified plasmids.

The extent of late protein expression from plasmids pEPΔI, pEPΔII and pEPΔE2A was determined by Western blot analysis and is discussed in section 4.4.

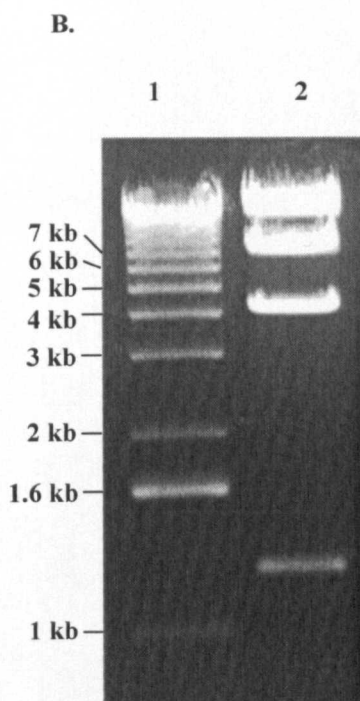
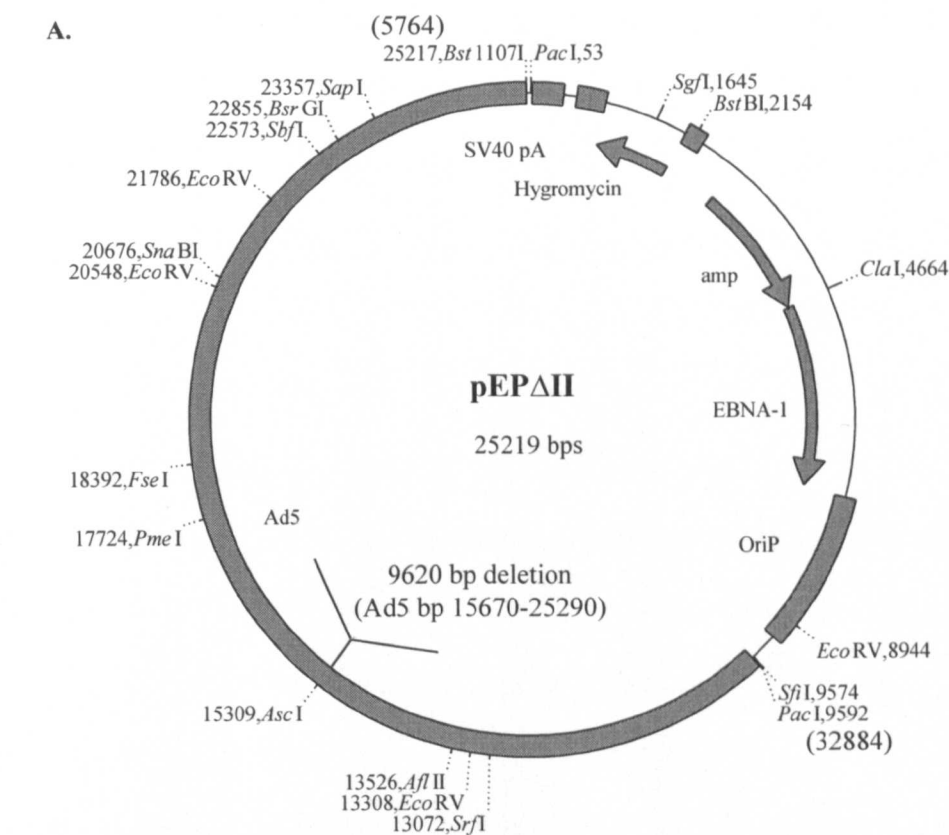


Figure 5.3. Plasmid pEPΔII.

A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992).

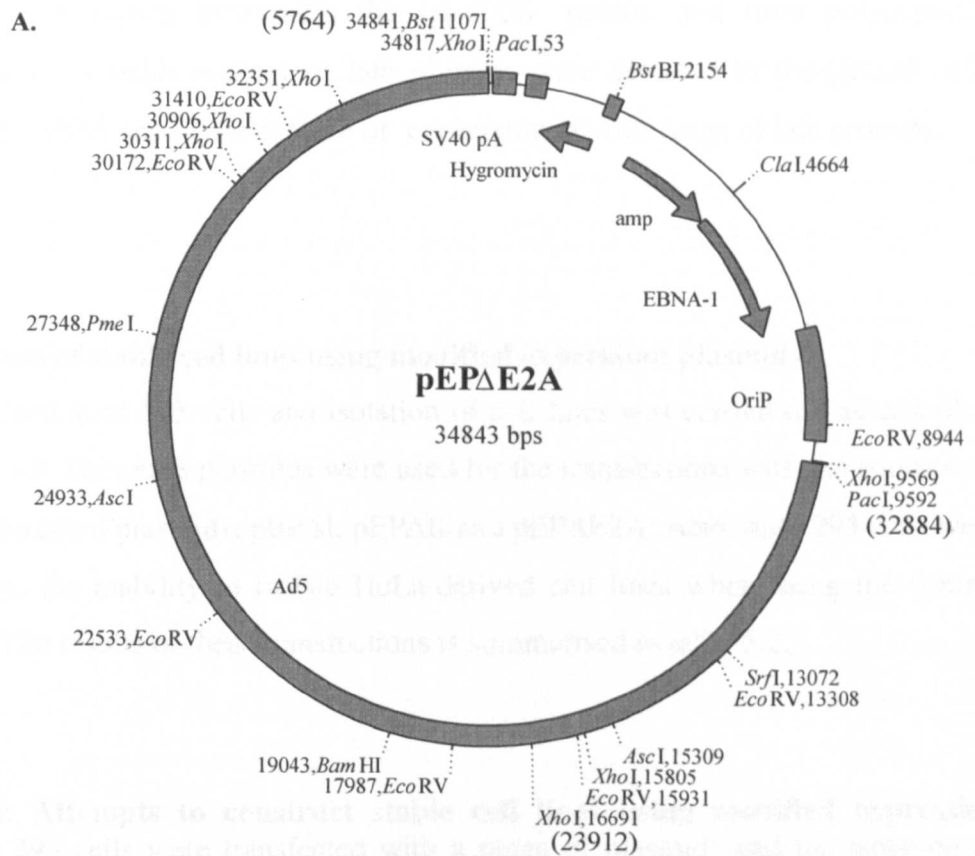
Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene; Ad5, adenovirus sequence.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pEPΔII.

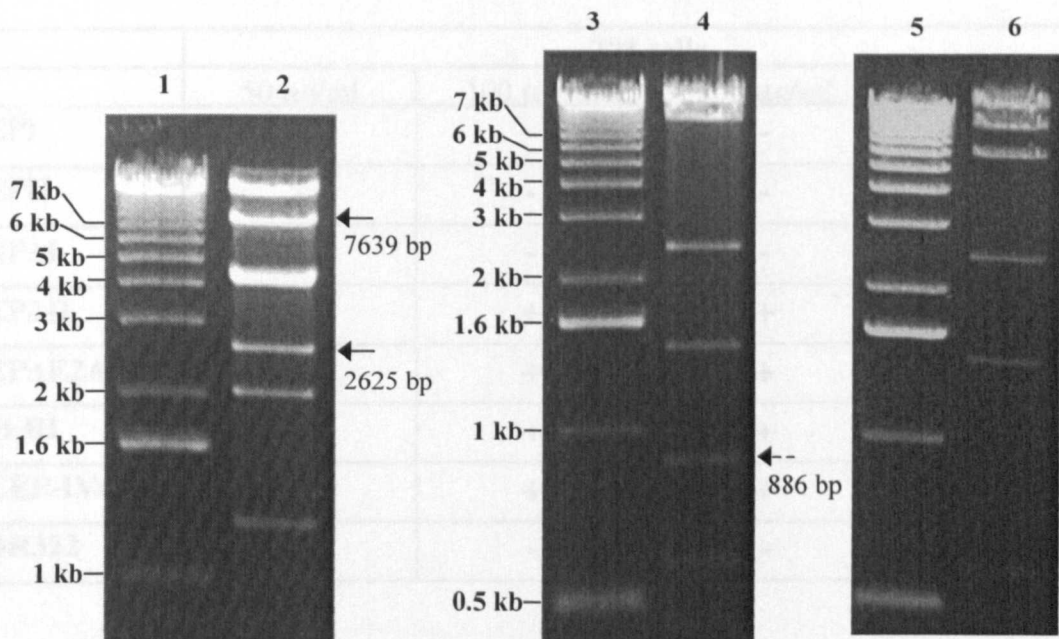
Figure 5.4. Plasmid pEPΔE2A.

A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1. Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene, Ad5, adenovirus sequence.

B. 1 % agarose gels visualised by ethidium bromide staining. Lanes 1, 3 and 5, 1 kb ladder. Lane 2, *EcoRV* digest of pEPΔE2A. Lane 4, *XhoI* digest of pEPΔE2A. Lane 6, *XhoI* digest of pEPII. The arrows indicate diagnostic bands used to determine insert orientation. The band showing presence of E2A mutation is indicated by a broken arrow.



B.



(figures 4.6 and 4.7). Briefly, pEP Δ I was shown to express the L4-100K and fibre polypeptides; pEP Δ II expressed the hexon, fibre and IIIa polypeptides and plasmid pEP Δ E2A was shown to express the L4-100K, penton and fibre polypeptides, Although only a small number of late proteins were detected in transfected cells, plasmid pEP Δ E2A should be capable of expressing the full range of late proteins.

5.5. Isolation of stable cell lines using modified expression plasmids.

Transfection of 293 cells and isolation of cell lines was carried out as described in section 5.3. The same plasmids were used for the transfections with the addition of the three modified plasmids; pEP Δ I, pEP Δ II and pEP Δ E2A. Also, only 293 cells were used due to the inability to isolate HeLa-derived cell lines when using the control plasmids. The results of these transfections is summarised in table 5.2.

Table 5.2. Attempts to construct stable cell lines using modified expression plasmids. 293 cells were transfected with a range of plasmids and the isolation of stable cell lines was attempted over a range of hygromycin-B concentrations (50 – 300 μ g/ml). + indicates that a stable cell line was isolated. – indicates that a stable cell line was not isolated.

	293 cells			
	50 μ g/ml	100 μ g/ml	200 μ g/ml	300 μ g/ml
pEPI	-	-	-	-
pEPII	-	-	-	-
pEP Δ I	-	-	-	-
pEP Δ II	+	+	+	+
pEP Δ E2A	+	+	+	+
pgERI	+	+	+	+
pCEP-IVa2	+	+	+	+
pBR322	-	-	-	-

As was previously observed, the two control plasmids, pgERI and pCEP-IVa2, were stably maintained in 293 cells, whereas the two expression plasmids, pEPI and pEPII were not. Of the three modified plasmids both pEPΔII and pEPΔE2A gave rise to stable cell lines, but pEPΔI did not. The only difference plasmid pEPΔE2A has when compared to pEPI/II is that it has a frameshift mutation in the DBP coding region. Therefore, the fact that pEPΔE2A but not pEPI/II was capable of stable maintenance indicates that it was the expression of DBP that prevented stable cell lines from emerging, presumably because of toxicity. The DBP reading frame covers the region from bp 22445 to 24032; plasmid pEPΔI has a deletion that covers bp 13258 to 21563 and therefore retains the DBP reading frame. The deletion in plasmid pEPΔII covers the region from bp 15760 to 25290 and therefore does not contain the DBP reading frame. The presence of the DBP coding region in pEPΔI and its inability to be stably maintained further supports the suggestion that DBP is toxic to 293 cells.

A transient transfection assay was used to confirm that DBP was being expressed from the plasmids that were incapable of being stably maintained. Transfected cell proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed for DBP expression using an anti-DBP monospecific antiserum (figure 5.5). Both plasmid pEPII and pEPΔI were found to express DBP when transfected into 293 cells, but as predicted plasmids pEPΔII and pEPΔE2A did not. Thus confirming that the expression of DBP correlates with the inability of a plasmid to be stably maintained.

The cell line created by plasmid pEPΔE2A, termed 293-EP, was chosen for further analysis as it should be capable of expression of the full range of late proteins. Also, the major proteins recognised by the available antisera were L4-100K, penton and fibre, of which only fibre is expressed by plasmid pEPΔII. This would make analysis of expression from the pEPΔII derived cell line a more difficult task.

5.6. Analysis of the 293-EP cell line.

5.6.1. Analysis of late protein expression in 293-EP cells.

Protein samples from 293-EP cells were taken 14 and 28 days post transfection and analysed by Western blotting using antisera AbJLB1 and AbR1/89. No

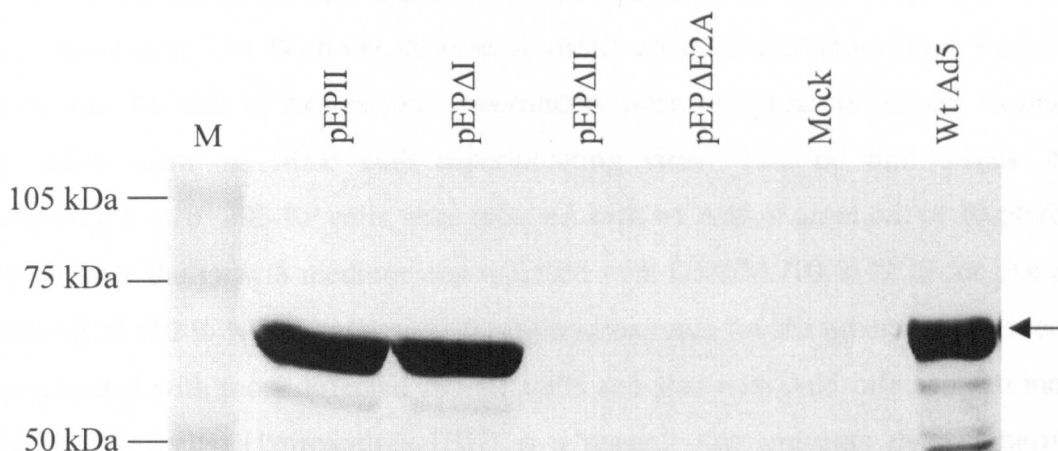


Figure 5.5. Expression of DBP in transiently transfected cells.

10 μ g of each plasmid was transfected into 4×10^6 293 cells in a 60 mm dish using calcium phosphate precipitation. Cells were left for 48 hours before protein extracts were made as described in section 2.3.5.1.

Protein extracts from 1×10^6 cells were analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with anti-DBP antiserum. The plasmid transfected is indicated above the relevant lane with Ad5-infected cell extract used as a positive control. Positions of molecular weight markers (M) are indicated on the left, the arrow on the right indicates the position of DBP.

cells that were infected with Ad5, suggesting that the presence of early viral proteins was not sufficient to activate the MLP. Analysis of the crude protein extracts with anti-DBP antibody (Figure 5.5B) confirmed the activation of Ad5 early protein DBP from the infecting virus, which would suggest that all the other early proteins were also expressed.

The previous result also revealed that the two earliest viral proteins IVa1 and IX, were not expressed in cells infected with Ad5 (Figure 5.5B). The IVa2 protein is a

expression of late proteins could be detected (data not shown), even when protein from 2×10^6 cells was loaded on the gel. This is approximately twice the amount of protein that was loaded to detect late protein expression after transient transfection. 293-EP cells were also analysed by immunofluorescence microscopy using AbR1/89 and anti-L4 100K antisera but, once again, no late protein expression could be detected (data not shown).

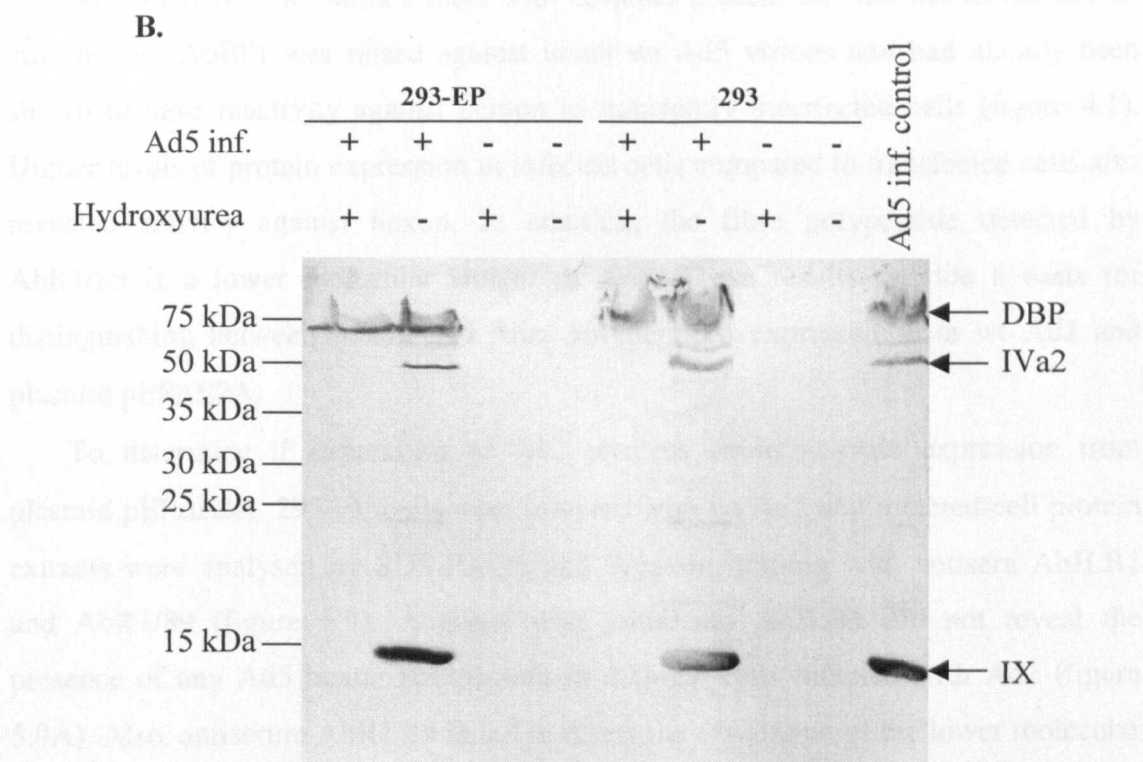
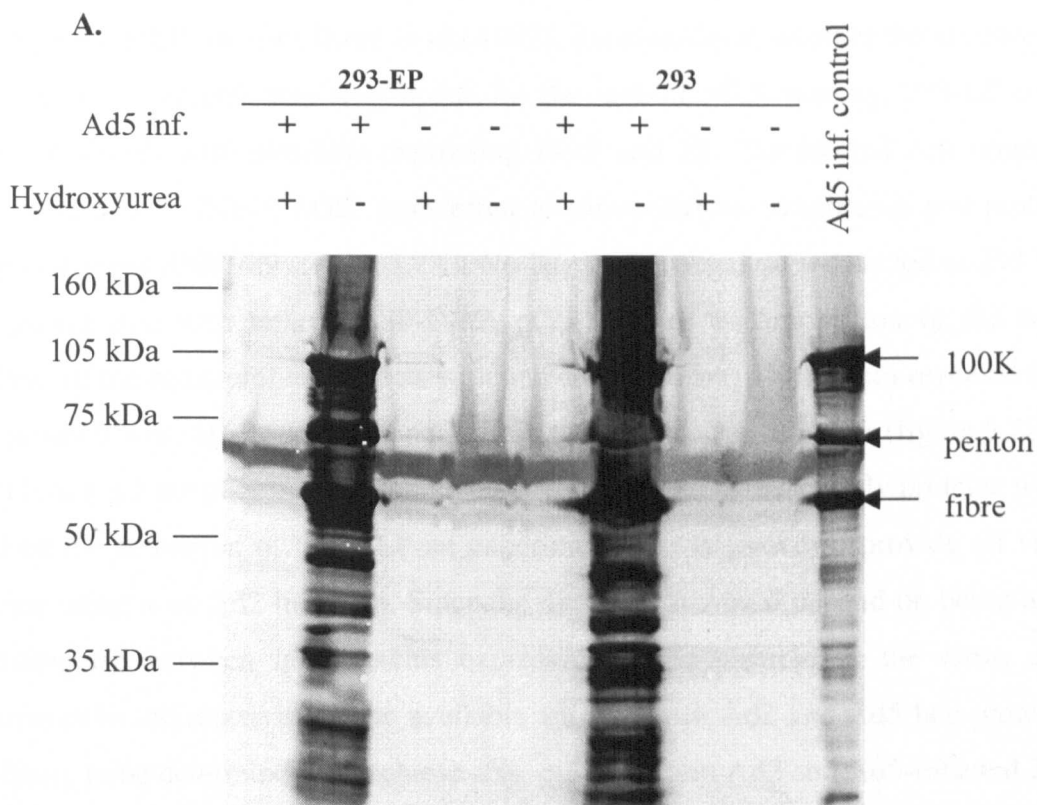
Research has shown that regulation of the MLP may be affected by several viral proteins (section 3.4). With the absence of virally encoded activators being a possible reason for the lack of expression, experiments were designed to attempt to induce expression from the MLP with superinfecting virus. Two 60 mm dishes, each containing 4×10^6 293-EP cells were infected with wt Ad5 at an m.o.i. of 10 pfu/cell. After 1 hour the growth medium was replaced with DMEM /10 % NCS for one dish and DMEM /10 % NCS containing 10 mM hydroxyurea for the other. This procedure was repeated with mock-infected 293-EP cells and also with Ad5-infected and mock-infected 293 cells. Hydroxyurea (HU) is a reagent that prevents the synthesis of deoxyribonucleotides by specifically inhibiting ribonucleoside diphosphate reductase. Therefore, treating infected cells with HU prevents viral DNA replication and late protein synthesis. Any late protein expression observed in these cells would be due to the MLP /MLTU construct present on the expression plasmid being activated by early proteins expressed by the virus. Cells were grown for 48 hours before protein extracts were made and separated by SDS-PAGE with the protein from 1×10^6 cells loaded per lane. Proteins were then transferred to nitrocellulose membranes and probed using antiserum AbR1/89 (figure 5.6A).

100K, penton and fibre expression was detected in infected 293 cells but not in HU-treated / infected 293 cells. This shows that treatment of infected cells with 10 mM HU effectively blocked viral DNA replication and late protein expression. However, no evidence of late protein expression could be detected in infected 293-EP cells that were treated with HU, suggesting that the presence of early viral proteins was not sufficient to activate the MLP. Analysis of the same protein extracts with anti-DBP antibody (figure 5.6B) confirmed the expression of the early protein DBP from the infecting virus, which would suggest that all the other early proteins were also expressed.

The previous result also revealed that the two delayed early proteins, IVa2 and IX, were not expressed in cells treated with HU (figure 5.6B). The IVa2 protein is a

Figure 5.6. Attempted induction of late protein expression in 293-EP cells using wt Ad5 infection.

Protein extracts from 293-EP and 293 cells, analysed on a 10 % (A) or 15 % (B) (w/v) polyacrylamide-SDS gel followed by Western blotting with polyclonal antiserum AbR1/89 (A) or anti – DBP, anti – IVa2, anti – pIX antisera (B). The use of Ad5 superinfection and / or 10 mM hydroxyurea treatment is indicated above the lane. Positions of molecular weight markers are indicated on the left, with arrows on the right indicating the position of viral proteins.



known transcriptional activator of the MLP (Tribouley *et al.*, 1994; Lutz and Kedinger, 1996) and IX has been shown to activate TATA containing promoters, including the MLP, *in vitro* (Lutz *et al.*, 1997). To investigate whether the absence of either of these proteins was responsible for the lack of MLP activity, 293-EP cells were transfected with plasmids expressing IVa2 and IX. Transfected cell proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed using antiserum AbR1/89 (figure 5.7A). No late proteins could be detected in 293-EP cells transfected with either pCEP-IVa2, pCMV-IX or a combination of the two. Analysis of the same protein extracts with anti-IVa2 and anti-IX antisera revealed that both proteins were effectively expressed from their respective plasmids (figure 5.7B).

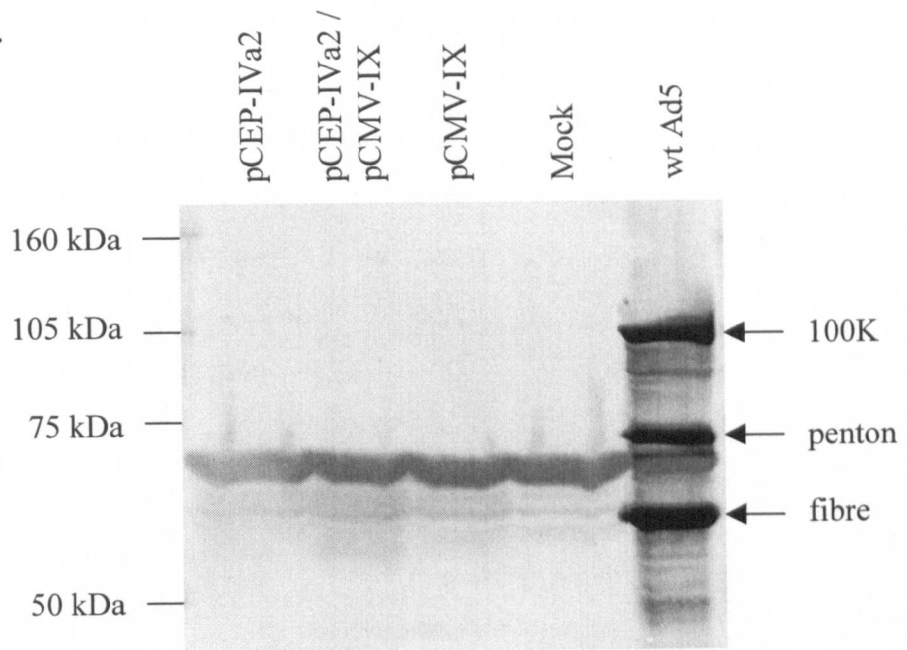
To rule out the possibility that both early proteins and delayed early proteins were required for activation of the MLP an experiment was designed to provide all viral proteins using a wt Ad2 infection. Since the experiment would depend on being able to distinguish between late proteins expressed by the plasmid or the virus, any differences in the reactivity of the available antisera with Ad2 and Ad5 late proteins had firstly to be determined. To achieve this, proteins from Ad2 and Ad5-infected 293 cells were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with AbJLB1, AbBF1 or AbR1/89 (figure 5.8). From these results it is clear that both AbJLB1 and AbBF1 react with epitopes present on Ad5 hexon but not on Ad2 hexon. AbBF1 was raised against intact wt Ad5 virions and had already been shown to have reactivity against penton in transiently transfected cells (figure 4.1). Higher levels of protein expression in infected cells compared to transfected cells also revealed activity against hexon. In addition, the fibre polypeptide detected by AbR1/89 is a lower molecular weight in Ad5. These results provide a basis for distinguishing between hexon and fibre polypeptides expressed from wt Ad2 and plasmid pEPΔE2A.

To determine if expression of Ad2 proteins could activate expression from plasmid pEPΔE2A, 293-EP cells were infected with wt Ad2 and infected-cell protein extracts were analysed by SDS-PAGE and Western blotting with antisera AbJLB1 and AbR1/89 (figure 5.9). Analysis with antiserum AbJLB1 did not reveal the presence of any Ad5 hexon polypeptide in 293-EP cells infected with Ad2 (figure 5.9A). Also, antiserum AbR1/89 failed to detect the expression of the lower molecular

Figure 5.7. Attempted induction of late protein expression in 293-EP cells using pIX and IVa2 expression plasmids.

10 µg of plasmid pCEP-IVa2 (figure A7) and / or pCMV-IX (Caravokyri and Leppard, 1995) were transfected into 4×10^6 293-EP cells in 60 mm dishes by calcium phosphate precipitation. Cells were grown for 48 hours before protein extracts were made as described in section 2.3.5.1. Protein extracts from 1×10^6 cells were analysed on 10 % (A) or 15 % (B) (w/v) polyacrylamide-SDS gels followed by Western blotting with polyclonal antiserum AbR1/89 (A) or anti -DBP, anti - IVa2 and anti - pIX antisera (B). Plasmid(s) transfected into 293-EP cells are indicated above the relevant lane, with wild type Ad5-infected cell extract used as a positive control. Positions of molecular weight markers (M) are indicated on the left, with arrows on the right indicating the position of viral proteins.

A.



B.

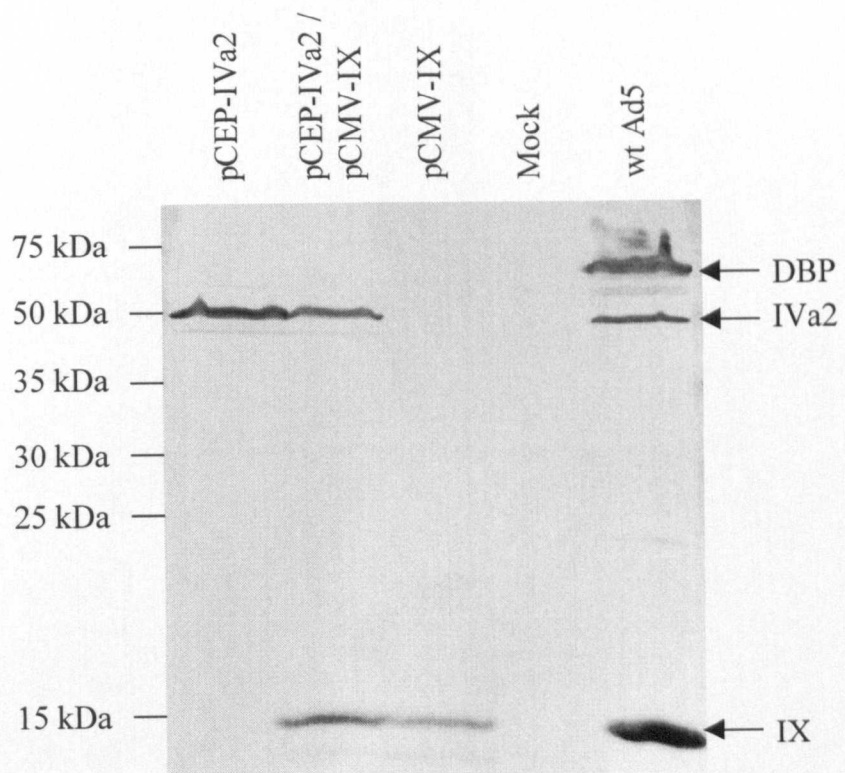
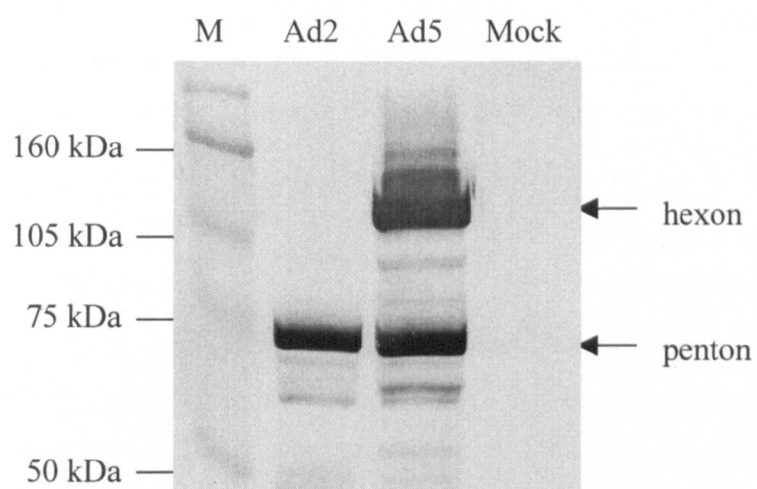


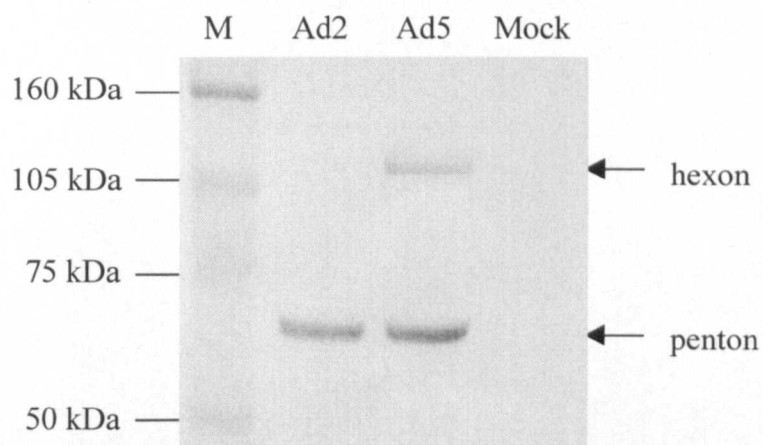
Figure 5.8. Differences in reactivity of antisera with Ad2 and Ad5 proteins.

4×10^6 293 cells in 60 mm dishes were infected with Ad2 or Ad5 at an m.o.i. of 10 pfu/cell and proteins extracted 24 hours later. Protein extracts from 5×10^5 cells were analysed on 10 % (w/v) polyacrylamide-SDS gels followed by Western blotting with polyclonal antiserum AbJLB1 (A), AbBF1 (B) or AbR1/89 (C). Positions of molecular weight markers (M) are indicated on the left, with arrows on the right indicating the position of viral proteins.

A.



B.



C.

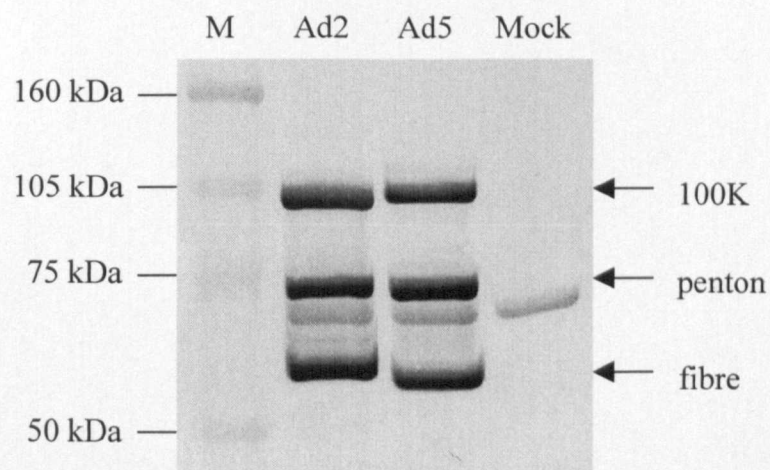
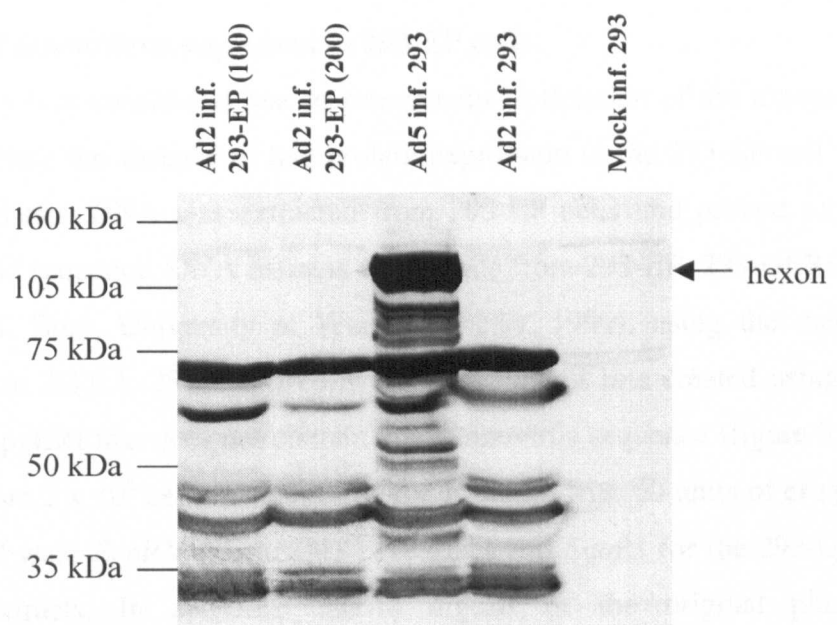


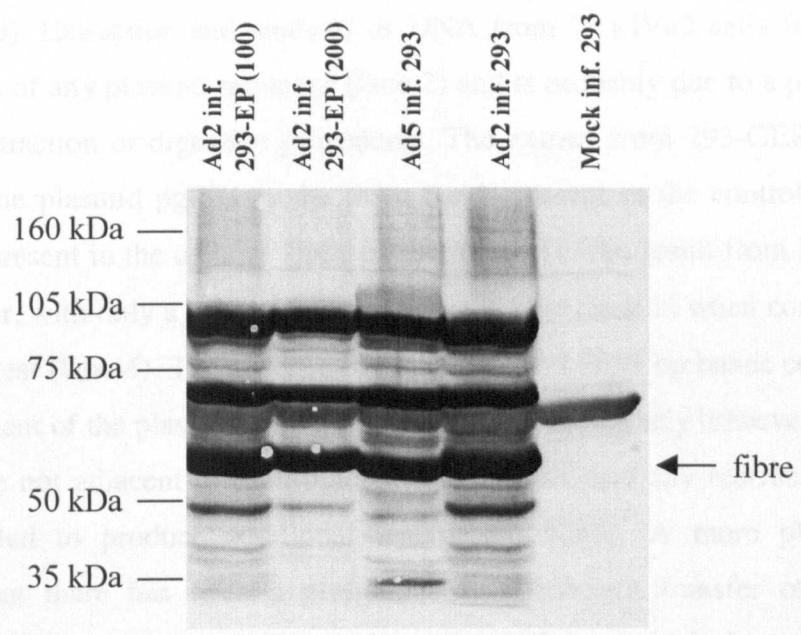
Figure 5.9. Attempted induction of late protein expression in 293-EP cells using wt Ad2 infection.

4 x 10⁶ 293-EP cells in 60 mm dishes were infected with wt Ad2 at an m.o.i. of 10 pfu/cell. 293 cells were also infected with Ad2 and Ad5 to provide controls. Protein extracts were made 48 hours post-infection and protein from 1 x 10⁶ cells was analysed on a 10 % (w/v) polyacrylamide-SDS gel followed by Western blotting with polyclonal antiserum AbJLB1 (A) or AbR1/89 (B). The cell type and Ad serotype used is indicated above the relevant lane. 293-EP (100) and 293-EP (200) indicates cells grown in the presence of 100 or 200 µg/ml hygromycin-B. Positions of molecular weight markers are indicated on the left, with arrows on the right indicating the position of viral proteins.

A.



B.



weight form of the Ad5 fibre polypeptide, with protein extracts from 293-EP cells containing only the higher molecular weight Ad2 form (figure 5.9B).

5.6.2. Detection of adenovirus sequences in 293-EP cells.

The possibility was considered that rearrangement or deletion of the expression plasmid might explain the absence of late protein expression in the 293-EP cell line. To test this hypothesis, DNA was extracted from 293-EP cells and probed for the presence of plasmid sequence. DNA extracts were made from 293-EP, 293-GERI and 293-IVa2 cells (S. Brey, University of Warwick thesis, 1999), using the method described in section 2.3.6.1. 293-GERI cells are a stable cell line created using the expression vector pgERI that does not contain any adenovirus sequence (figure 3.12). DNA extracted from 2×10^6 cells was digested for 16 hours with 60 units of enzyme. The enzymes used were *EcoRV* for the 293-EP extract and *EcoRI* for the 293-GERI and 293-IVa2 extracts. In addition, control digests of the original plasmid preparations were carried out as controls. Digested samples were separated on a 0.7 % agarose gel and analysed by Southern blotting using nick-translated pEPΔE2A as a probe (figure 5.10). Extraction and analysis of DNA from 293-IVa2 cells failed to show the presence of any plasmid sequence (lane 2) and is probably due to a problem with the DNA extraction or digestion procedures. The extract from 293-GERI cells clearly contains the plasmid pgERI as the three bands present in the control digest (lane 6) are also present in the cellular DNA extract (lane 3). The result from 293-EP cells is not as clear, with only a subset of bands being visible (lane 1) when compared to the plasmid digest (lane 4). The absence of the 12377 and 7639 bp bands could be due to rearrangement of the plasmid after transfection. This is unlikely however as the two fragments are not adjacent to each other in the plasmid and any rearrangement would be predicted to produce additional unexpected bands. A more plausible explanation is that there has been a problem with inefficient transfer of DNA fragments to the nylon membrane, a hypothesis supported by a degree of smearing in the area of the blot expected to contain the absent fragments.

Figure 5.10. Southern analysis of DNA sequences present in 293-EP cells.

DNA extracts from 293-EP, 293-GERI and 293-IVa2 cells were analysed on an 0.7 % agarose gel followed by Southern blotting using nick-translated pEPΔE2A as a probe. Plasmids used in the construction of the cell lines were also included as markers. Fragment sizes (in bp) are indicated.

Panel A. 14 day (336 hour) exposure.

Lane 1, 293-EP DNA extract from 2×10^6 cells, *EcoRV* digest.

Lane 2, 293-IVa2 DNA extract from 2×10^6 cells, *EcoRI* digest.

Lane 3, 293-GERI DNA extract from 2×10^6 cells, *EcoRI* digest.

Panel B. 1 day (24 hour) exposure.

Lane 4, Plasmid pEPΔE2A (50 ng), *EcoRV* digest.

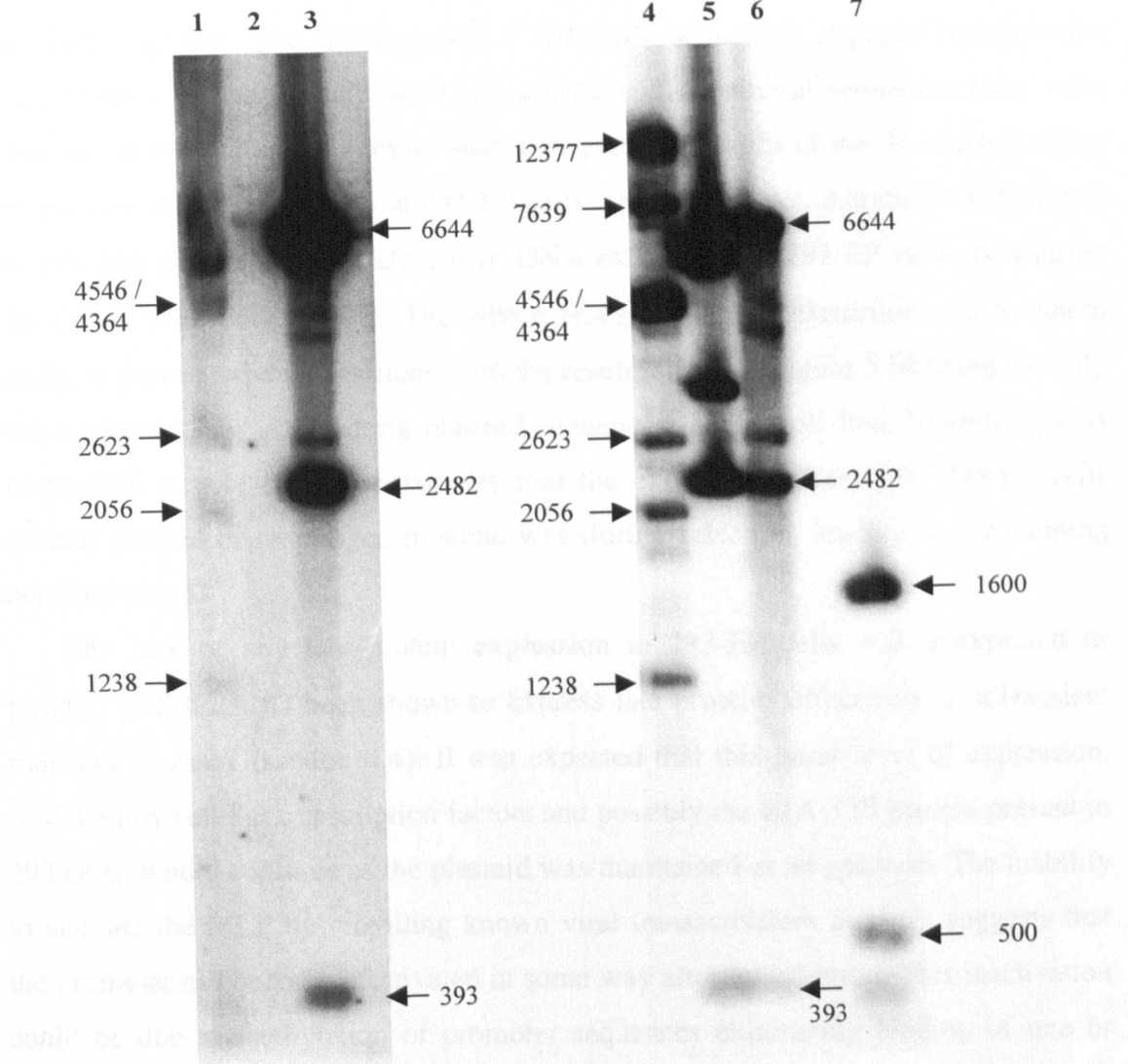
Lane 5, Plasmid pCEP-IVa2 (50 ng), *EcoRI* digest.

Lane 6, Plasmid pgERI (50 ng), *EcoRI* digest.

Lane 7, 1 kb ladder.

A.

B.



5.7. Discussion.

The initial problems encountered in creating stable cell lines with plasmids pEPI and pEPII were due to 293 cells being unable to tolerate DBP expression, presumably because of toxicity. This problem was resolved by introducing a frameshift mutation into the DBP reading frame and thus abolishing its expression. However, results from the subsequent experiments with the modified plasmid, pEPΔE2A, indicate that there is another fundamental problem in achieving expression of adenovirus late proteins in a stable cell line. The functionality of the EBV sequences required for episomal maintenance of the plasmid has been proven and adenoviral sequences have been detected in the stable cell lines created. However, the results of the Southern analysis of plasmid sequences present in 293-EP cells are inconclusive. Although the majority of plasmid pEPΔE2A was detected in DNA extracts from 293-EP cells, two larger fragments were undetectable. The whole process of DNA extraction and Southern analysis proved to be inconsistent, with the results shown in figure 5.10 being the only successful attempt at detecting plasmid sequences in any cell line. Therefore, it is impossible to rule out the possibility that the expression plasmid in 293-EP cells became deleted or rearranged in some way during selection, leading to it becoming non-functional.

The lack of any late protein expression in 293-EP cells was unexpected as plasmid pEPΔE2A has been shown to express late proteins effectively in a transient transfection assay (section 4.4). It was expected that this basal level of expression, mediated by cellular transcription factors and possibly the E1A-13S protein present in 293 cells, would continue as the plasmid was maintained as an episome. The inability to activate the MLP by providing known viral transactivators *in trans* suggests that the promoter has become inactivated in some way after transfection. This inactivation could be due to methylation of promoter sequences eliminating binding of one or more essential transcription factors, a process that has already been shown to regulate expression of the Ad2 E2A gene *in vitro* (Langner *et al.*, 1984; Hermann and Doerfler, 1991). In this case, downregulation of the E2A promoter is due to the methylation of specific 5' CCGG 3' sequences present in two AP2 sites. Methylation of these sequences prevents the AP2 transcription factor from binding to the DNA site and forming the transcription-activating complex. The adenovirus MLP contains one 5' CCGG 3' sequence which overlaps the UPE (upstream promoter element) which is

the USF transcription factor binding site (underlined) 5' GGCCACGTGACCGG 3'. Methylation of this sequence could possibly prevent USF binding to the UPE and therefore inhibit activation of the MLP. This possibility could be tested using methylation sensitive restriction enzymes and Southern blotting, provided greater reliability could be achieved with detecting recovered plasmid DNAs.

**Chapter 6 : Construction of a regulated system
for adenovirus type 5 late protein expression.**

Chapter 6 : Construction of a regulated system for adenovirus type 5 late protein expression.

6.1. Introduction.

Regulating recombinant protein production in mammalian cells has become an integral part of protein functional analysis. Having the ability to control when and how much of a particular protein is expressed makes it possible to study temporal and concentration-dependent effects on a host system and also allows the expression of toxic proteins.

One commonly used method to regulate transgene expression is the use of a metallothionein promoter. The metallothioneins are a highly conserved family of small, cysteine-rich proteins which bind heavy metals and are thought to be involved in heavy-metal detoxification and homeostasis (Hamer, 1986). They have been found to be inducible by heavy metals such as zinc, copper and cadmium, this induction being due to increased transcription of the gene (Durnam and Palmiter, 1981; Karin *et al.*, 1984).

The sheep metallothionein Ia (sMT-Ia) promoter is ideal for the regulated expression of toxic proteins as it is inducible but produces only low levels of basal expression, unlike the mouse metallothionein I promoter. This is due to the sMT-Ia promoter not containing a basal level expression sequence motif (Peterson and Mercer, 1986; Peterson *et al.*, 1988; Peterson and Mercer, 1988). Comparison of the sMT-Ia promoter sequence with the promoters of metallothionein genes from other species identified a number of conserved regions which may be important in the regulation of the sMT-Ia gene by heavy metals (Peterson and Mercer, 1986). These conserved regions are indicated in figure 6.1. Found between -21 and -27 is the TATA box (element a). Element b, found between -40 and -54, is highly conserved between MT genes (Stuart *et al.*, 1985) and has been implicated in the regulation of the mouse MT-I gene by heavy metals (Carter *et al.*, 1984; Stuart *et al.*, 1984). Sequences homologous to these mouse and human heavy metal response elements (MREs) also appear a further five times in the sMT-Ia promoter (elements c – g). The promoter has been shown to be induced by zinc, copper and cadmium (Peterson and

TGGAGAATGTAAGGAAGCTGGGGCGACAGAAAGCTGAGTTTCGCTGCTGGGCTGTTACAGGAGAACTAGAGACTCTGTTCAAAGTCCAGGGTGGGGCTG
 -501
 TGGGAGGAAATATTAGGAAAGCGGGGTTTCGGGGGATAGGTGGTGAAGCTCACATCCATCAGGGTCTCTGCACACGACACAGGGGCTCCAGCCAAAGCCTG
 -401
 GGATGTGAGCACGAGGCTCGGATTGCGCATGAGCTCTGGGAAAGGGTGAAGCAAGAGTTGCGGGGCAGGGAAGACTGCGAGGACTCAGGGAC
 -301
 TGGGTTCCCGTAAACACCGATGACTGCCACATTGTGGAAGCTGGGAAGGGCGGGCAGGAATCTGGAGGCTACTTGTCTATTCTGGGACAAAAGTCCCT
 -201
 CCGCGTTGGGGCGAGTAGGGGACGGAGGCGTTTCGGTTCGCGACGGAGCCAGCCGCTTCCGGGAATCTTGGCGCTCGGCCGCGTGGTGTCTCACCGC
 -101
 CCGACCCGGGTGCAGCGGCGAGCTCGGGTGCAGGCGGGGGCAGACCCCTCTGCGCCCCGCCCTCCTGTGGGTATATAATAGCGCTCGGCTCCTGGGCTCC
 -1
 d c b a

Figure 6.1. DNA sequence of the sMT-1a promoter region. The sequence depicted lies upstream from the transcription start site, which is located adjacent to the final nucleotide shown (-1). Several proposed regulatory elements within the promoter region which are discussed in the text are underlined and labelled a - i.

Genbank Accession No. X04626

Mercer, 1986), with addition of 100 μ M ZnSO₄ producing a 95-fold increase of sMT-Ia mRNA when compared to the basal level

6.2. Aims.

The results presented in chapter 5 indicated that there was a problem in activating the MLP when the expression plasmid was present in a stable cell line. Potentially, active expression was being selected against by virtue of the toxicity of one or more protein products. The aim of the work described here was to overcome this difficulty by developing a system for regulated expression of late proteins through the use of the sheep metallothionein-Ia promoter.

6.3. Construction and analysis of a regulated reporter plasmid.

In order to assess the optimum conditions for induction of the metallothionein promoter a reporter plasmid was constructed which contained the *lacZ* gene from *E. coli*, downstream of the sheep metallothionein promoter. The enzyme β -galactosidase encoded by the *lacZ* gene catalyses the hydrolysis of various β -galactosides. Its activity can be monitored histochemically by the cleavage of the substrate X-gal (5-bromo-4-chloro-3-indolyl β -D-galactoside; Alam and Cook, 1990) which is converted to an indigo blue derivative that is readily visible in cells.

To construct the reporter plasmid pMT-*lacZ*, the *lacZ* gene was excised from plasmid pcDNA3.1/*lacZ* (Invitrogen, figure A.6) by digestion with *KpnI* and *XbaI*. The resulting 3121 bp fragment was subsequently cloned into *KpnI* / *XbaI* digested pMT-CB6+ (Cook *et al.*, 1996; figure A.11). An *EcoRI* digest, generating bands of 4864, 3096 and 868 bp, was used to identify clones containing the insert (figure 6.2).

The optimum conditions for induction of the sMT-Ia promoter were determined by monitoring β -galactosidase expression from pMT-*lacZ* in 293 cells. 2×10^6 cells in 6-well plates were transfected with 2.5 μ g of pMT-*lacZ* using calcium phosphate

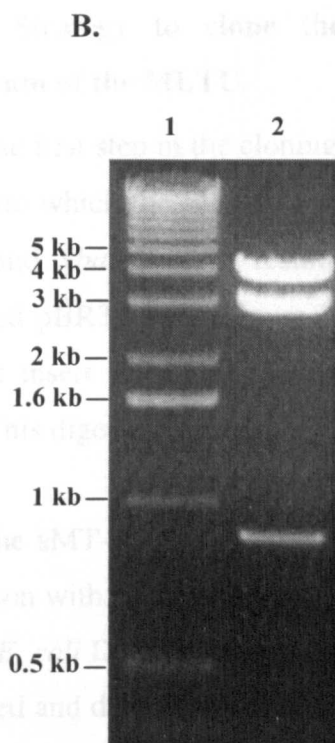
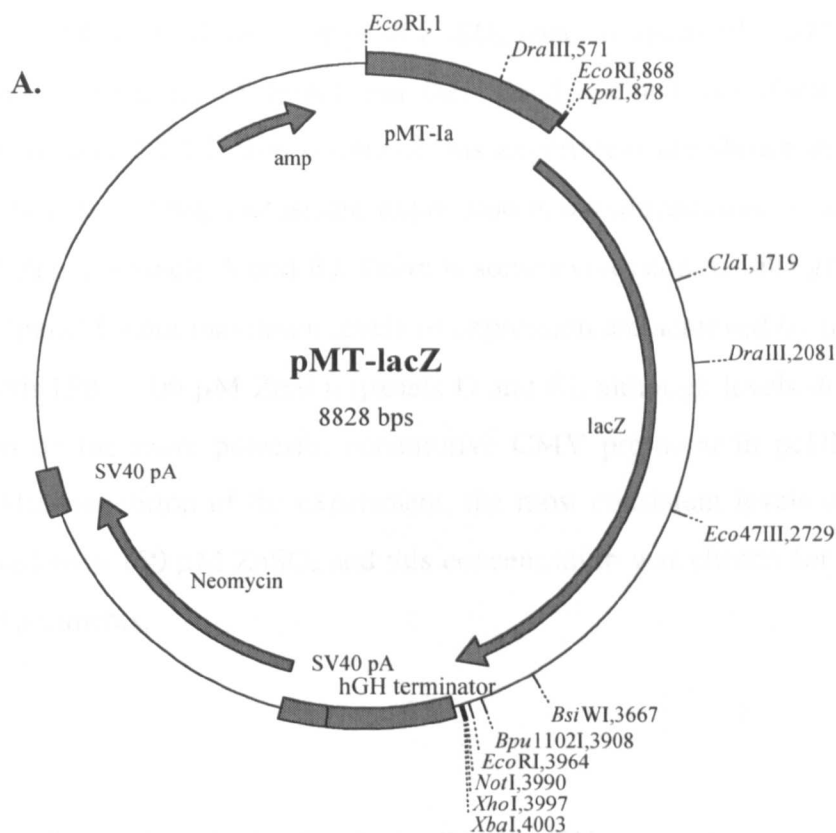


Figure 6.2. Plasmid pMT-lacZ.

Reporter plasmid containing the *lacZ* gene driven by a sheep metallothionein-1a promoter.

A. Plasmid map showing unique sites and sites of interest. pMT-1a, sheep metallothionein promoter. Amp, ampicillin resistance gene. Neomycin, neomycin resistance gene. SV40 pA, simian virus 40 polyadenylation signal.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RI digest of pMT-lacZ. The plasmid is predicted to yield fragments of 4864, 3096 and 868 bp.

precipitation. Cells were grown on glass coverslips to aid subsequent photography. Cells were grown for 24 hours after which time the growth medium was replaced with DMEM / 10 % NCS containing a range of ZnSO₄ concentrations (0 – 200 µM). Cells were grown for a further 48 hours and then fixed, stained and photographed as described in section 2.3.5.7. The results of this experiment are shown in figure 6.3. There is no evidence of β-galactosidase expression in uninduced cells or cells induced with 50 µM ZnSO₄ (panels A and B). There is some expression in cells grown in 100 µM ZnSO₄ (panel C) but maximum levels of expression are achieved by inducing the promoter with 150 – 200 µM ZnSO₄ (panels D and E), although levels did not reach those shown by the more powerful constitutive CMV promoter in pcDNA3.1/*lacZ* (panel F). After repetition of the experiment, the most consistent levels of induction were observed with 150 µM ZnSO₄ and this concentration was chosen for any further induction experiments.

6.4. Construction and analysis of an inducible MLTU expression cassette.

6.4.1. Strategy to clone the sheep metallothionein Ia (sMT-Ia) promoter upstream of the MLTU.

The first step in the cloning strategy was to create a subclone of pBRZ17I (figure 3.9) into which the sMT-Ia promoter could be inserted. pBRZ17I was digested with *PacI* and *XbaI* and the resulting 4844 bp fragment was cloned into *PacI* / *XbaI* digested pBR322PXP (figure A.12) creating plasmid pKW2S. Plasmids bearing the correct insert were determined by restriction enzyme analysis with *EcoRV* (figure 6.4). This digest generated the predicted bands of 5755, 2040 and 1238 bp.

The sMT-Ia promoter was removed from plasmid pMT-CB6+ (figure A.11) by digestion with *EcoRI*. The cohesive ends of the resulting 868 bp fragment were filled using *E. coli* DNA polymerase and the modified fragment cloned into *BstZ17I* / *PvuII* digested and dephosphorylated pKW2S. This cloning strategy was chosen as it placed the transcription start site and TATA box of the sMT-Ia promoter in approximately the same position as the equivalent excised MLP sequences (figure 6.5).

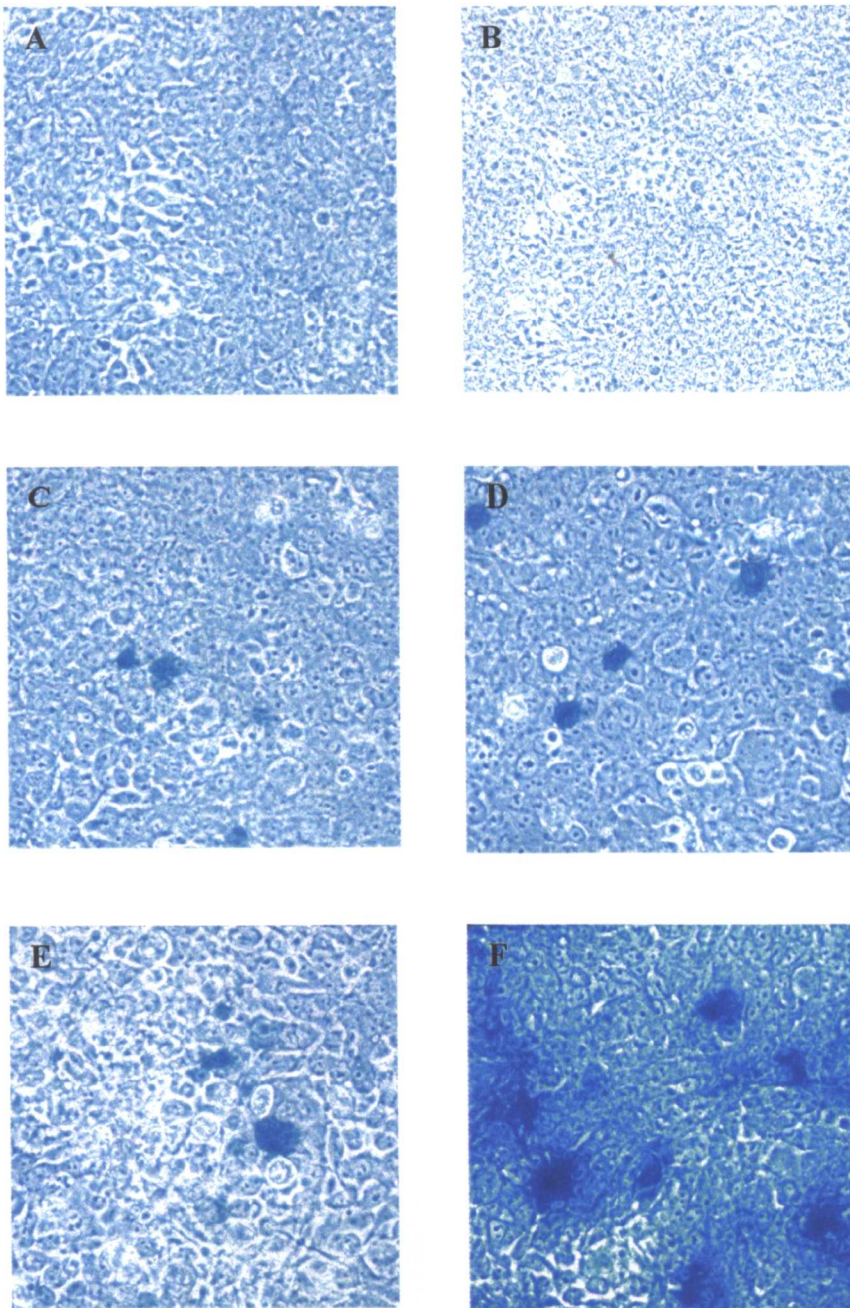
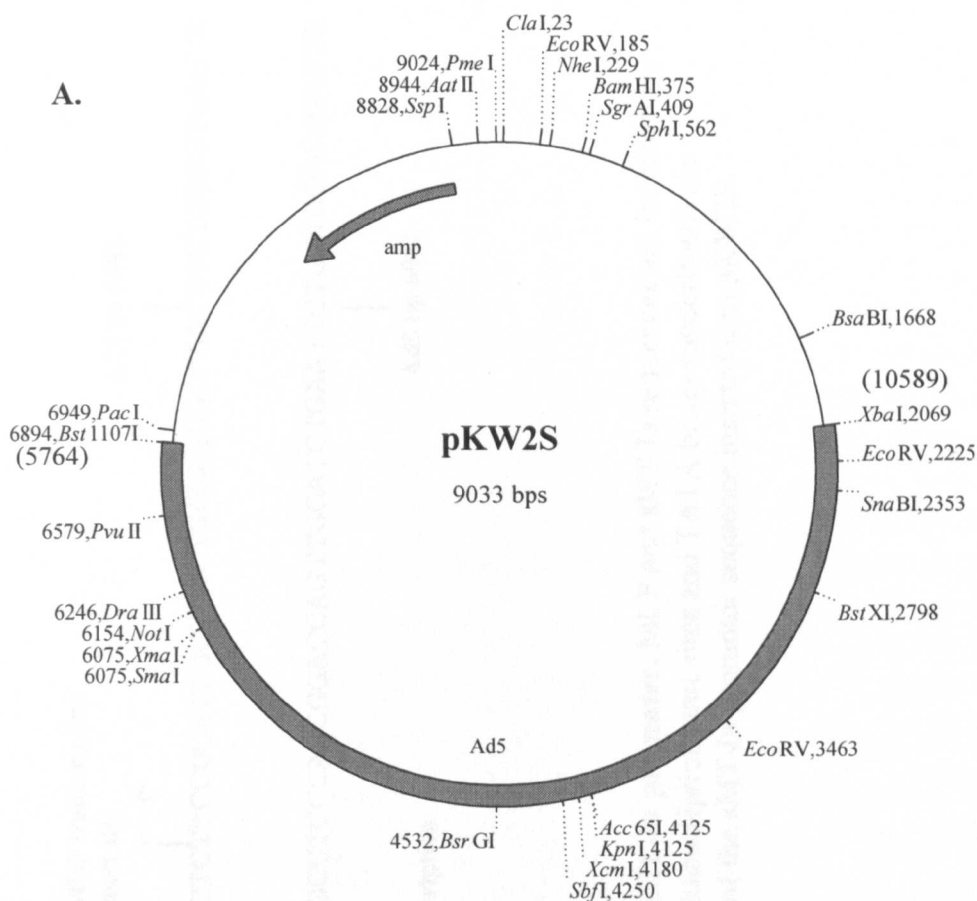


Figure 6.3. Induction of β -galactosidase expression in 293 cells. 293 cell monolayers transfected with pMT-*lacZ* and stained for β -galactosidase expression following induction with ZnSO_4 at various concentrations; **A**, uninduced control. **B**, 50 μM . **C**, 100 μM . **D**, 150 μM . **E**, 200 μM . **F**, control plasmid pcDNA3.1/*lacZ*. Magnification x 20.



B.



Figure 6.4. Plasmid pKW2S. Plasmid bearing Ad5 sequence from 5764 - 10589.

A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp, ampicillin resistance gene. Ad5, adenovirus sequence.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pKW2S.

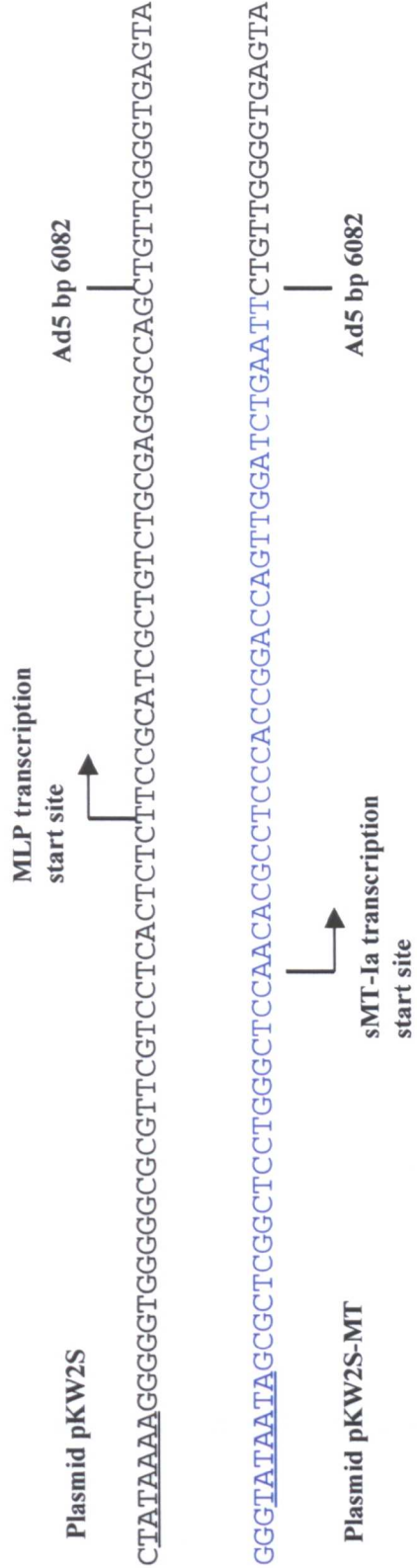


Figure 6.5. Replacement of the MLP with the sMT-Ia promoter. MLP and sMT-Ia sequences are indicated by black and blue text respectively. Positions of the transcription start sites and TATA boxes (underlined) are indicated. The figure depicts only a selected part of the sMT-Ia promoter sequence inserted into pKW2S.

The resulting plasmid, termed pKW2S-MT, was identified by restriction enzyme analysis with *Hind*III and *Dra*III (figure 6.6). The *Hind*III digest generated the predicted bands of 6388 and 3202 bp, confirming the presence of the insert. The orientation of the insert was confirmed by the presence of a 633 bp band after *Dra*III digestion. The insert in the alternative orientation would have produced a 902 bp band.

The viral sequence in plasmid pKW2S-MT was extended rightward to the *Xba*I site at Ad5 bp 28592 by cloning the 18007 bp *Xba*I fragment from pEPΔE2A (figure 5.4) into the *Xba*I site of pKW2S-MT, creating plasmid pMT-*Xba*I. Plasmids containing the insert in the correct orientation were determined by restriction enzyme analysis with *Eco*RV (figure 6.7). Diagnostic junction fragments of 7639 and 3183 bp showed that the insert was present and in the correct orientation. Insert in the alternative orientation would have produced bands of 9367 and 1455 bp. The *Xba*I fragment from pEPΔE2A was used for this cloning step so that the expression plasmid would contain the DBP frameshift mutation, shown previously to be required to permit stable plasmid maintenance in 293 cells (chapter 5).

The viral sequence in plasmid pMT-*Xba*I was extended to the right end of the viral genome by cloning the 7332 bp *Spe*I / *Cla*I fragment from pAdMLTU (figure 3.10) into *Spe*I / *Cla*I digested pMT-*Xba*I, creating plasmid pMT-MLTU. Plasmids bearing the correct insert were determined by restriction enzyme analysis with *Eco*RV (figure 6.8). This digest generated the predicted bands of 7639, 6312, 4586, 4546, 2623, 2374, 2056 and 1238 bp.

6.4.2. Construction of an episomal expression plasmid containing the sMT-Ia / MLTU cassette.

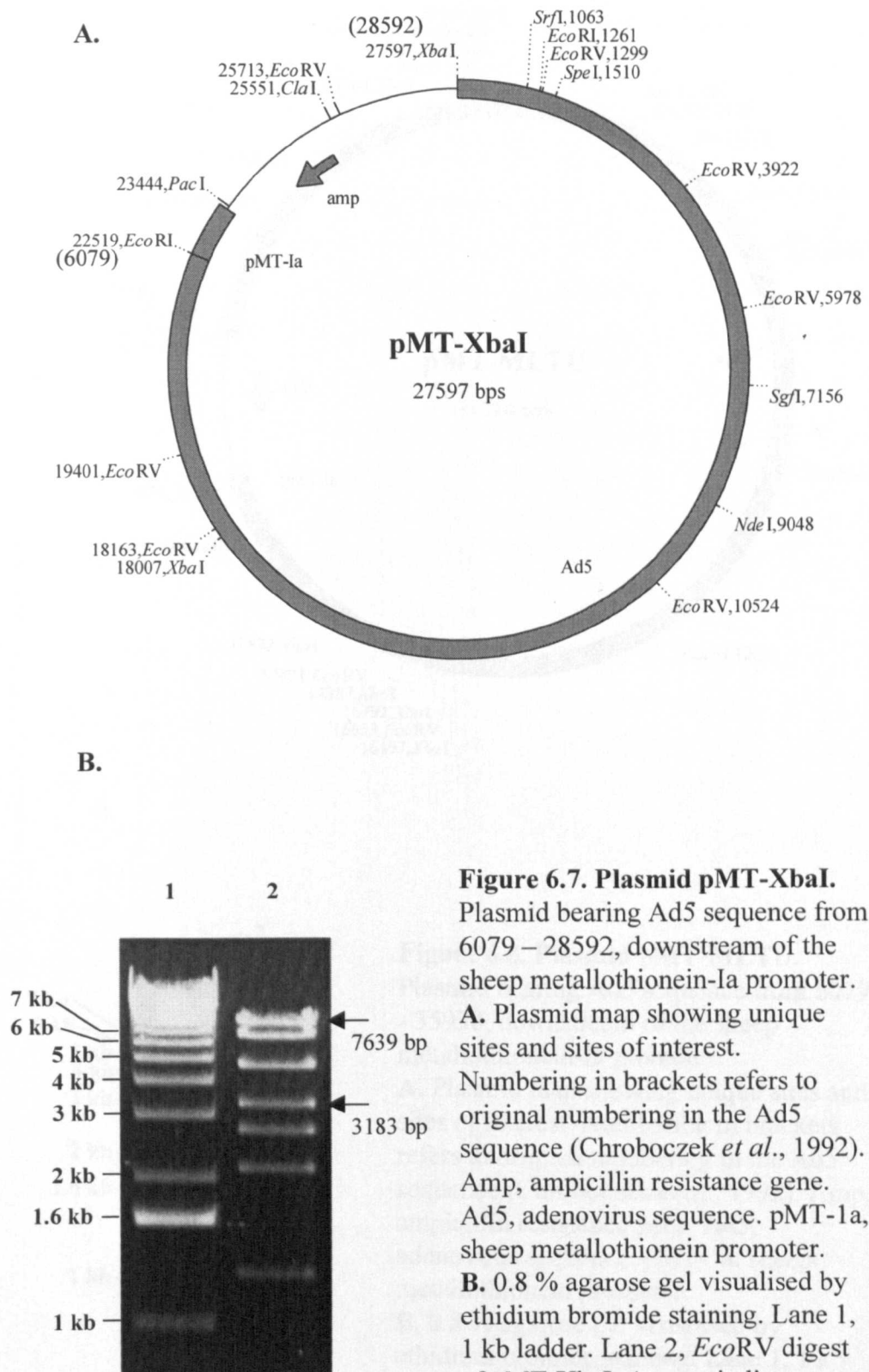
The sMT-Ia / MLTU cassette was excised from plasmid pMT-MLTU by digestion with *Pac*I. The resulting 25861 bp fragment was cloned into *Pac*I digested and dephosphorylated pgERI (figure 3.12), creating plasmid pMT-EP. Plasmids containing the insert in the desired orientation were determined by restriction enzyme analysis with *Xho*I and *Eco*RV (figure 6.9). The 6236 bp diagnostic junction fragment

Figure 6.6. Plasmid pKW2S-MT. Plasmid bearing Ad5 sequence from 6079 – 10589, downstream of the sheep metallothionein-1a promoter.

A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp, ampicillin resistance gene. Ad5, adenovirus sequence. pMT-1a, sheep metallothionein promoter.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Hind*III digest of pKW2S-MT.

1.4 % agarose gel visualised by ethidium bromide staining. Lane 3, 1 kb ladder. Lane 4, *Dra*III digest of pKW2S-MT. Arrow indicates diagnostic band used to determine insert orientation.



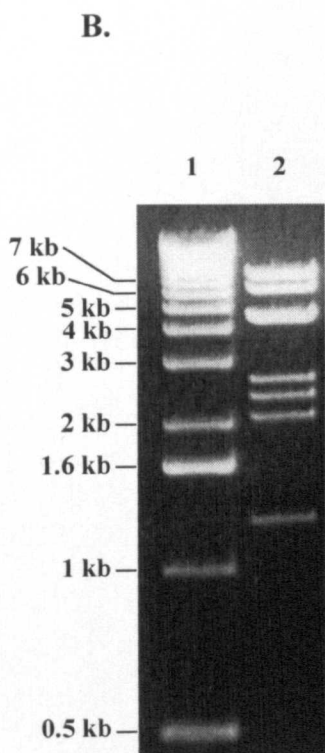
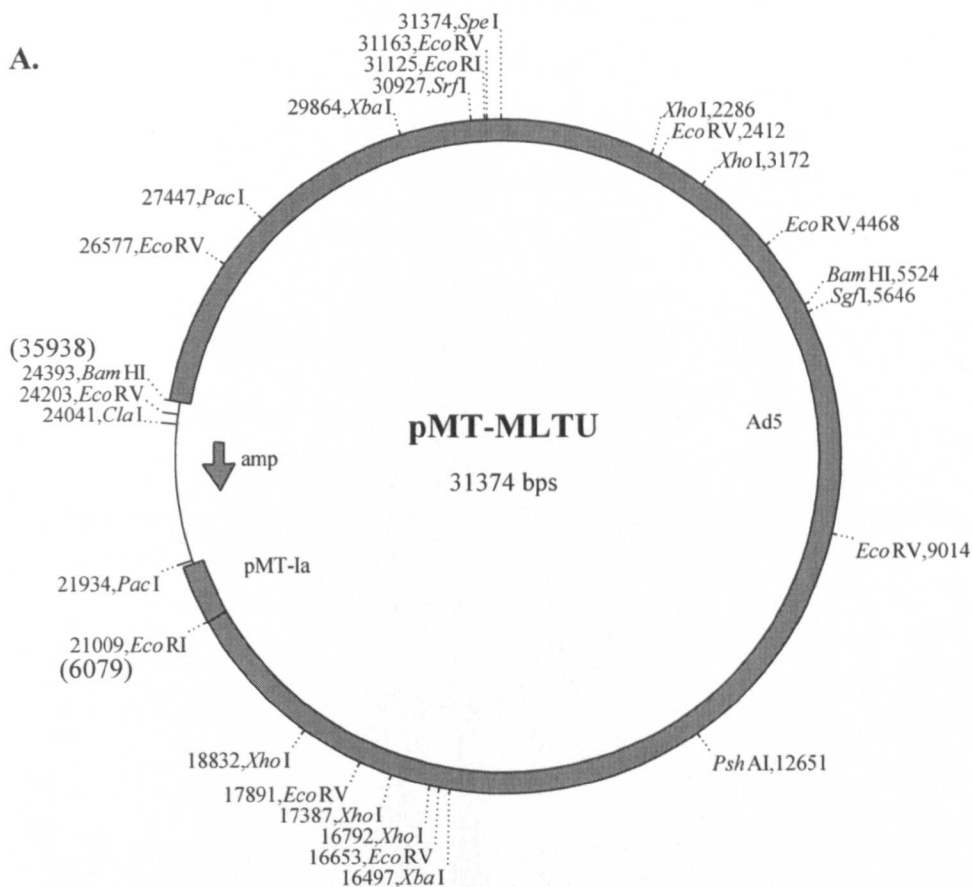


Figure 6.8. Plasmid pMT-MLTU.

Plasmid bearing Ad5 sequence from 6079 - 35938, downstream of the sheep metallothionein-1a promoter.

A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Amp, ampicillin resistance gene. Ad5, adenovirus sequence. pMT-1a, sheep metallothionein promoter.

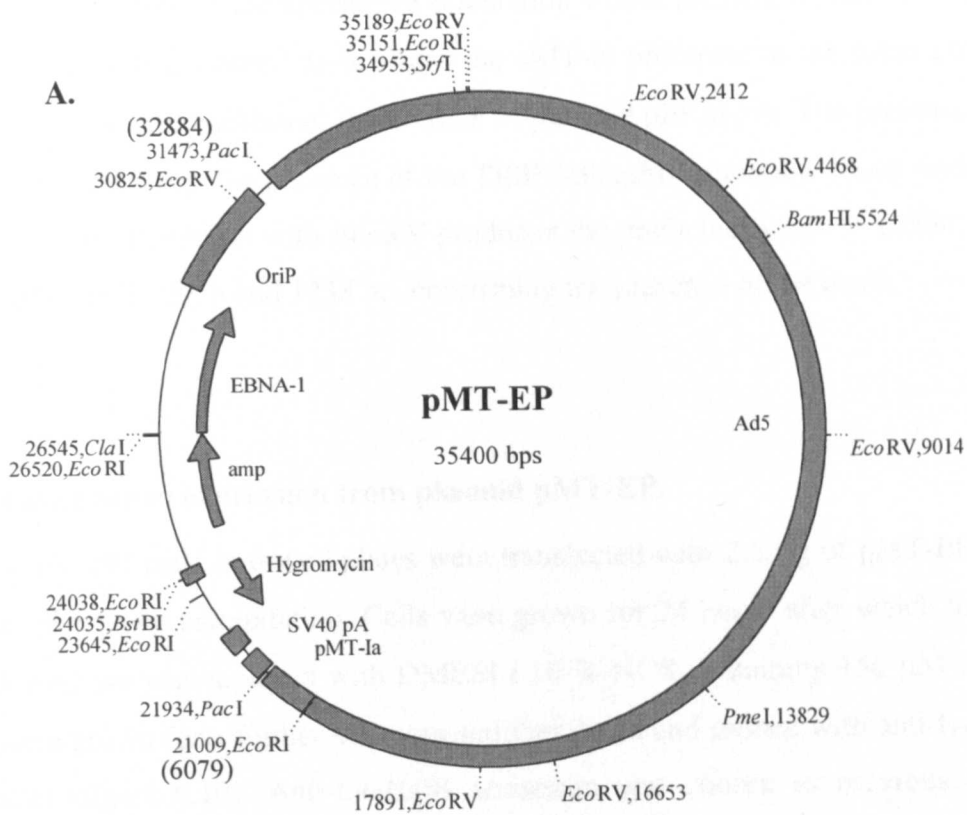
B. 0.8 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *Eco*RV digest of pMT-MLTU.

Figure 6.9. Plasmid pMT-EP. Episomal expression plasmid bearing Ad5 sequence from 6079 – 32884, downstream of the sheep metallothionein-Ia promoter.

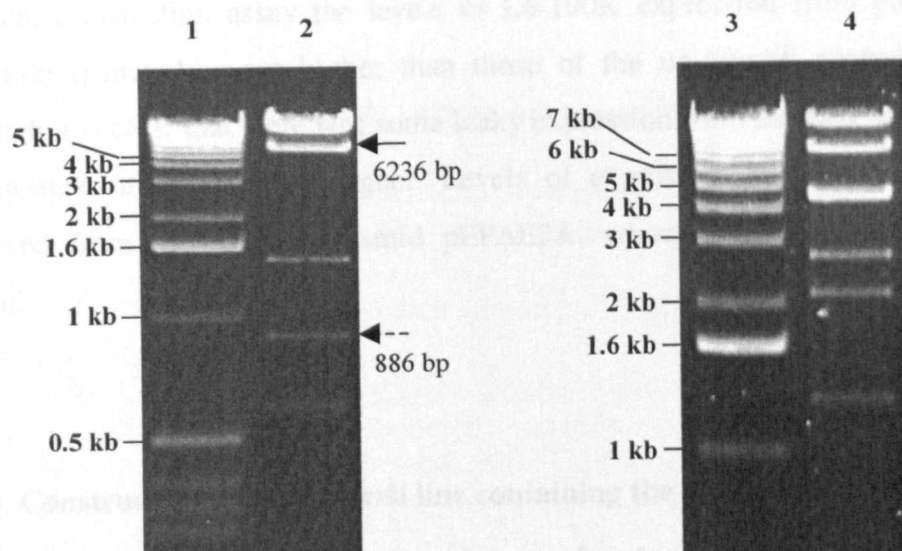
A. Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992).

Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; pMT-1a, sheep metallothionein promoter; OriP, EBV latent origin of replication; EBNA-1. Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene.

B. 0.8 % agarose gels visualised by ethidium bromide staining. Lane 1 and lane 3, 1 kb ladder. Lane 2, *Xho*I digest of pMT-EP. Lane 4, *Eco*RV digest of pMT-EP. The arrow indicates diagnostic band used to determine insert orientation. The band showing the presence of the E2A mutation is indicated by a broken arrow.



B.



present after *Xho*I digestion showed that the insert was present and in the correct orientation. Insertion in the alternative orientation would produce a band of 3127 bp. This orientation was chosen as it places the sMT-Ia promoter in the same position, relative to the vector backbone, as the MLP in plasmid pEPΔE2A. The presence of an 886 bp band confirms the presence of the DBP frameshift mutation which creates an extra *Xho*I site. Digestion with *Eco*RV produced the predicted bands of 12394, 7639, 4546, 4364, 2623, 2056 and 1238 bp, confirming the presence of the insert.

6.4.3. Late protein expression from plasmid pMT-EP.

2 x 10⁶ 293 cells in 6-well plates were transfected with 2.5 µg of pMT-EP using calcium phosphate precipitation. Cells were grown for 24 hours after which time the growth medium was replaced with DMEM / 10 % NCS containing 150 µM ZnSO₄. Cells were grown for a further 48 hours and then fixed and probed with anti-L4-100K antiserum (figure 6.10). Anti-L4-100K antiserum was chosen as previous control experiments had shown this to be the most effective serum of those available for detecting adenovirus proteins expressed from plasmid pEPΔE2A in an immunofluorescence assay. Also included were an uninduced control, a mock transfection and a positive control transfection of plasmid pEPΔE2A. In this transient transfection assay the levels of L4-100K expression from pMT-EP after induction (panel A) were higher than those of the uninduced control (panel B), although it is clear that there was some leaky expression from the sMT-Ia promoter in the absence of the inducing agent. Levels of expression were lower than those observed from the control plasmid pEPΔE2A, whose expression is under MLP control.

6.4.4. Construction of a stable cell line containing the sMT-Ia / MLTU construct.

10 µg of plasmid pMT-EP was used to transfect 4 x 10⁶ 293 cells in a 60 mm dish using calcium phosphate precipitation. At 48 hours post-transfection the cells were passaged 1:3 and after a further 24 hours the growth medium was replaced with

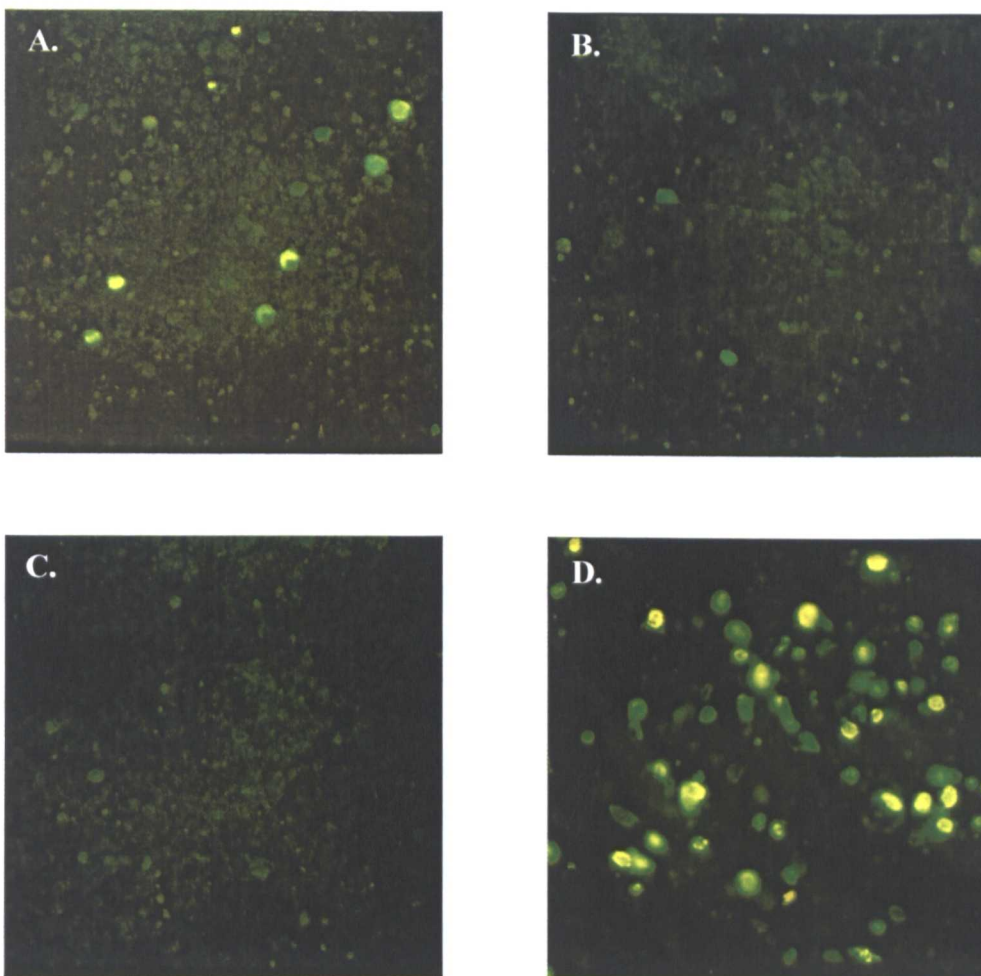


Figure 6.10. Induction of L4-100K late protein expression from plasmid pMT-EP.

Panel A. 293 cells transfected with expression plasmid pMT-EP, induced 24 hours post-transfection by addition of 150 μM ZnSO_4 to growth medium.

Panel B. 293 cells transfected with expression plasmid pMT-EP, uninduced control.

Panel C. Mock transfected 293 cells after addition of 150 μM ZnSO_4 .

Panel D. 293 cells transfected with expression plasmid pEP Δ E2A. All cells were probed with anti-L1-100K antiserum 48 hours post-transfection and photographed using a 20 X objective.

DMEM / 10 % NCS containing 200µg / ml hygromycin-B. The growth medium was replaced every 3 days and cells passaged 1:3 when confluent.

After 28 days, 1×10^6 cells were seeded in 6-well plates and grown for 24 hours before replacing the growth medium with DMEM / 10 % NCS / 150 µM ZnSO₄. Cells were grown for a further 48 hours before being fixed and probed with anti-L4-100K antiserum. However, no expression of L4-100K could be detected using this method. Similarly, no late protein expression could be detected in cell extracts using Western blotting with antiserum AbR1/89 (data not shown).

6.4.5. Verification of expression vector functionality.

The EBV replicon vector backbone sequences used throughout this study derived from the same source as those used previously to create cell lines expressing individual late proteins (Caravokyri and Leppard, 1995; S. Brey, University of Warwick thesis, 1999; A. Arslanoglu, University of Warwick thesis, 1999). Moreover, they were shown to be capable of conferring stable resistance to selection in this study (chapter 5). However, in order to confirm that they were capable of supporting stable expression of heterologous genes, a reporter plasmid containing a GFP expression cassette was constructed. The green fluorescent protein (GFP) reporter gene is derived from the bioluminescent jellyfish *Aequorea victoria*, and upon exposure to blue light (excitation peaks at 395 and 470) fluoresces with green light emission (between 509 and 540 nm) (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). The version of the gene used here has been modified to produce more rapid and intense fluorescence, with a mutation at residue 65 of serine to threonine, which results in a single excitation peak of 490 nm (Heim *et al.*, 1995). GFP has a major advantage over other routinely used reporter genes, in that there is no need to fix cells or for a substrate, making analysis easier and faster.

In order to construct plasmid pEP-GFP, the expression vector sequences were excised from plasmid pMT-EP by digestion with *PacI* and a GFP expression cassette was excised from plasmid pHGFP-S65T (Clontech, figure A.10) by digestion with *MluI* and *BamHI*. The cohesive ends of this 2263 bp fragment were converted to blunt ends using the Klenow filling method, as were the ends of the 9539 bp fragment produced by *PacI* digestion of pMT-EP. These two fragments were subsequently

ligated together to create plasmid pEP-GFP. Restriction enzyme analysis using EcoRV and HindIII (figure 6.11) produced a 2248 bp fragment confirming the insert was in the desired orientation. An insert in the alternative orientation would have produced a 1307 bp fragment. The insert orientation chosen was the one that would put the CMV promoter of the GFP cassette in the same position relative to the vector backbone as the sMT-Ia and major late promoters used in the other expression plasmids described in this study.

Plasmid pEP-GFP was used to transfect 4×10^6 293 cells in 60 mm dishes using calcium phosphate precipitation. At 48 hours post-transfection the cells were passaged 1:3 and after a further 24 hours the growth medium was replaced with DMEM / 10 % NCS containing 200 μ g / ml hygromycin-B. The growth medium was replaced every 3 days and cells passaged 1:3 when confluent. Cells were grown on glass coverslips and samples removed at 1, 15 and 42 days post transfection and assayed for expression of GFP using the method described in section 2.3.5.4. (figure 6.12). Expression of GFP can be seen at all times assayed post transfection. The number of fluorescent cells increases between 1 and 15 days post-transfection, as selection increases their proportion within the population. There is little reduction in fluorescence intensity over the time-course of the experiment although there is considerable heterogeneity in expression level, particularly at 42 days post-transfection, when it might be expected that all cells would harbour and express the plasmid. Overall, these results show that the EBV expression vector sequences are capable of allowing stable maintenance and prolonged expression of heterologous genes in 293 cells.

6.5. Discussion.

The sMT-Ia promoter was used successfully to regulate the expression of both a *lacZ* reporter gene and the adenovirus MLTU. However, this regulation of expression was only possible in a transient transfection assay. When plasmid pMT-EP was used to create a stable cell line no expression of late proteins could be detected, with or without the presence of ZnSO₄ in the growth medium to induce the promoter. These results mirror those obtained with plasmid pEP Δ E2A, in that it would also only

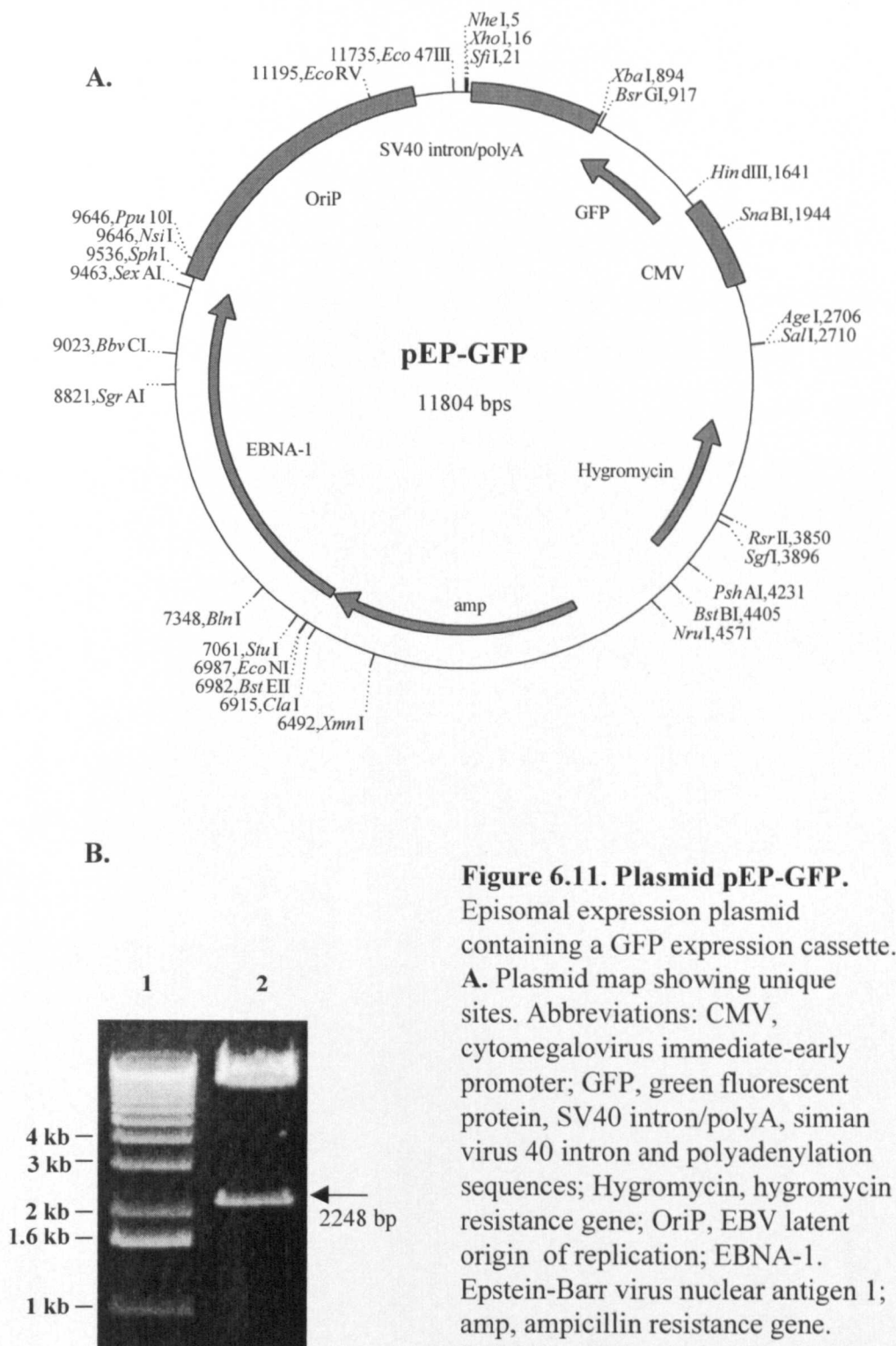


Figure 6.11. Plasmid pEP-GFP.

Episomal expression plasmid containing a GFP expression cassette.

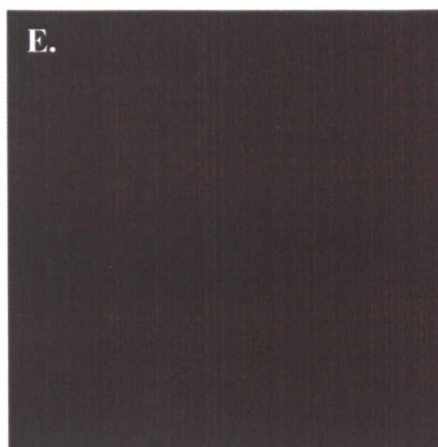
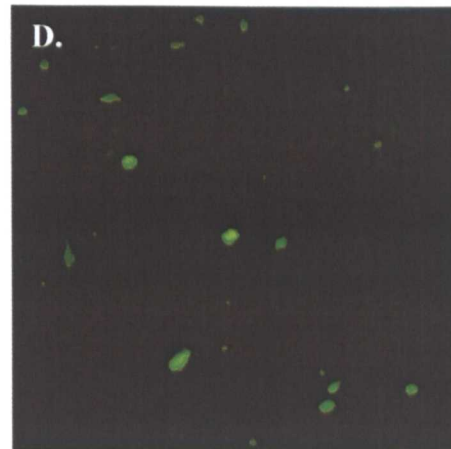
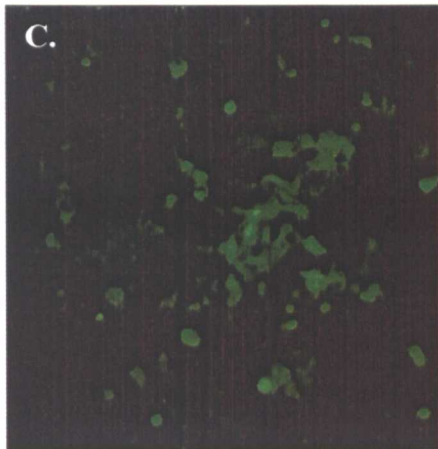
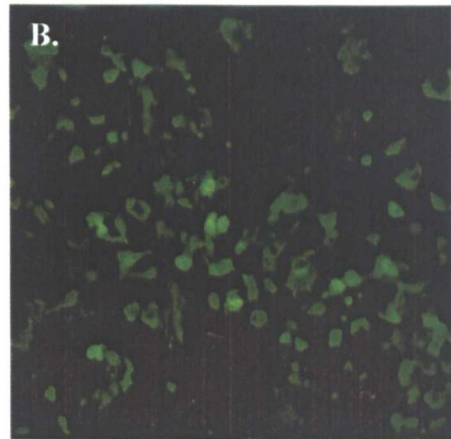
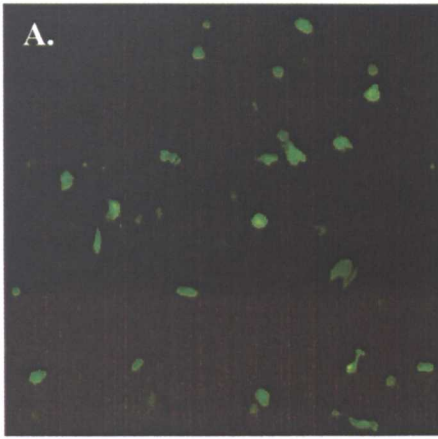
A. Plasmid map showing unique sites. Abbreviations: CMV, cytomegalovirus immediate-early promoter; GFP, green fluorescent protein, SV40 intron/polyA, simian virus 40 intron and polyadenylation sequences; Hygromycin, hygromycin resistance gene; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene.

B. 1 % agarose gel visualised by ethidium bromide staining. Lane 1, 1 kb ladder. Lane 2, *EcoRV* and *HindIII* digest of pEP-GFP. Arrow indicates diagnostic band used to determine insert orientation.

Figure 6.12. Persistence of expression from an episomal plasmid in 293 cells.

293 cells were transfected with episomal reporter plasmid pEP-GFP. Cells were put under positive selection 3 days post transfection and passaged 1:3, when confluent. Cells were assayed for GFP expression 1 day (panel A), 15 days (panel B) and 42 days (panel C) post-transfection. Panel D shows 293 cells transfected with pHGFP-S65T, 1 day post-transfection and is used as a positive control for GFP fluorescence. Panel E shows 293 cells transfected with plasmid pgERI.

Magnification x 20.



express late proteins in transient transfections. The results of the experiment with the episomal expression vector carrying the GFP cassette show that the inability to create a functional stable cell line is not due to the EBV replicon. The EBV sequences used to create plasmid pEP-GFP were taken straight from plasmid pMT-EP, and were then used successfully to create a stable GFP-expressing cell line. Taken together, the results from this chapter and chapter 5 strongly support the suggestion that it is changes to the adenovirus sequences subsequent to transfection that are in some way responsible for the inability of the plasmid to express late proteins in a stable cell line.

Chapter 7 : Discussion.

Chapter 7 : General Discussion.

7.1. Introduction.

Of the numerous vectors investigated for use in gene therapy, those based on human adenoviruses have proved efficient for delivery of genes both *in vitro* and *in vivo*. Adenovirus vectors have many advantages over other viral vectors for use in gene therapy. They have a wide tropism and can infect both dividing and non-dividing cells. They have a good safety profile, are easy to manipulate *in vitro* and can be grown to high titres. Despite these advantages, low transgene-carrying capacity and the lack of long-term persistence of gene expression, due to immune mediated clearance of the vector, remain problems to be solved.

The aim of the work presented here was to achieve improvements over currently existing vectors for gene therapy, through the construction of cell lines capable of supporting the growth of viruses with late gene deletions. Deletion of late gene regions would increase transgene carrying capacity and reduce the expression of potentially immunogenic viral proteins, hopefully leading to increased transgene expression. The majority of the work presented here focused on overcoming problems associated with expressing late proteins in eukaryotic cells as discussed below.

7.2. Problems associated with the expression of Ad5 late proteins.

The Ad5 MLTU was successfully cloned and late proteins expressed from the construct in a transient transfection system. The initial attempts to isolate stable cell lines were unsuccessful due to the expression of the toxic E2A-DNA binding protein. Subsequent removal of DBP allowed the expression plasmid to be stably maintained in 293 cells. However, no late protein expression from the MLTU could be detected in these cell lines. Attempts to activate expression from the major late promoter by providing viral transactivating factors *in trans* also proved to be ineffective.

The ability of the expression plasmids used to express late proteins in a transient transfection but not in a stable cell line was the common outcome of the work described in both Chapters 5 and 6. There are several possibilities that may account for these observations. Perhaps the most obvious reason is that the plasmids became modified in some way during selection of the cell lines, most likely as a response to selective pressure resulting from late protein expression from the plasmid that occurred immediately after transfection. Detectable levels of late protein expression after transient transfection were not expected in the absence of the viral transactivating IVa2 protein but considerable expression was observed. The probable explanation for this is that after transfection, basal transcription from the MLP occurs. As any transfected cell will receive multiple copies of the plasmid, this initial late protein expression is at a high enough level to be detected by Western blot. However, this basal level of expression may be toxic to the cell and is suppressed shortly after transfection. This down-regulation of late protein expression could be due to either reduction in plasmid copy number, defects in post-transcriptional RNA processing or promoter methylation, as discussed below.

7.2.1. Reduction in plasmid copy number.

During the initial transfection procedure each cell will receive multiple copies of the expression plasmid. Multiple plasmids, all expressing low levels of late protein would be expected to produce a detectable level of expression. However, during selection of cell lines, plasmid copy number could decrease to a level where resistance to hygromycin-B is maintained but expression of late proteins is at a minimum, possibly due to toxicity effects of expressed proteins. In light of previous results, this hypothesis is unlikely. Previously, three cell lines have been created using the EBV replicon-containing plasmids that express the IX, L1 52/55K and IVa2 proteins (Caravokyri and Leppard, 1995; A. Arslanoglu, S. Brey, University of Warwick, PhD theses, 1999). All three cell lines were capable of expressing levels of protein equivalent to a wt infection, with no apparent reduction in the levels of expression over a prolonged period. Also, the levels of expression seen in transient transfection assays were similar to those seen in the stable cell lines. However, the proteins

expressed are not known to have any toxic effects on eukaryotic cells and expression would not be expected to be selected against.

The possibility that plasmid copy number decreases could be investigated by quantifying the number of copies per cell after transfection, then monitoring the cell population over time for any decrease. Quantification of Southern blots probed for plasmid sequences could be used to determine if copy number decreases over time. Alternatively, quantitative PCR (QPCR) could be used to determine the absolute number of plasmid copies per cell.

7.2.2. Promoter methylation.

The concept that sequence-specific promoter methylations and long-term gene inactivations might be causally related has been experimentally substantiated by investigations using a number of promoters (reviewed by Doerfler, 1983). In general, results have shown that the introduction of 5-methylcytosine residues into specific 5'-CG-3' (CpG) dinucleotides within a promoter inactivates the promoter. The modification of one or a few CpG sequences is sufficient to shut down a promoter, possibly by inhibiting DNA-protein interactions. For several transcription factors, evidence has been adduced that the methylation of their target sequences blocks their ability to bind specifically to these sequences (Kovesdi *et al.*, 1987; Hermann and Doerfler, 1991). This loss of interaction is likely to account for the transcriptional inhibition of methylated promoter sequences. In addition to this loss of factor binding, methylation may also promote the binding of repressor proteins. Recent studies have identified two proteins, MeCP1 and MeCP2, that bind specifically to methyl-CpG and repress methylated promoter activity *in vitro* as well as *in vivo* (Lewis *et al.*, 1992).

Methylation of CpG sequences present in the MLP promoter could account for the apparent shut-down of late protein expression in the stable cell lines. The MLP contains 8 CpG sequences, one of these is located in the USF transcription factor binding site, and another is adjacent to the DEF-B binding site. Methylation of these sequences could prevent the two transcription factors from binding to their specific sequences, thus preventing activation of the MLP. This would also explain why providing the IVa2 protein *in trans* within the cell lines did not result in activation of the promoter. Previous studies have shown that the transcription factor DEF-B is a

homodimer of the IVa2 protein (Tribouley *et al.*, 1994) and is responsible for maximal activation of the MLP at late times of infection (Jansen-Durr *et al.*, 1988; 1989). Binding of the USF protein is also required for activation of the MLP, and one could hypothesise that the lack of binding of these two factors would lead to complete inactivation of the MLP, as appears to be the case in the cell lines constructed in these studies.

Restriction enzymes that are sensitive to CpG methylation could be used to determine if the MLP becomes methylated during the selection of stable cell lines. Restriction enzymes *AciI*, *HhaI*, *NciI* and *PmlI* are sensitive to methylation and all have recognition sequences that are present in the MLP. Comparison of restriction digests of plasmid extracted from stable cell lines with those of unmethylated plasmid preparations would show if the MLP is methylated in the cell line.

In addition to methylation, chromatin-related transcriptional inactivation by histone deacetylases has been shown to down-regulate gene expression (Chen *et al.*, 1997; Pikaart *et al.* 1998). In the cell lines created, the EBNA-1 protein and OriP sequence interact to anchor the plasmid episome to the nuclear matrix (Krysan and Calos, 1993; Middleton and Sugden, 1992,1994) and this may make the plasmid sensitive to gene silencing by histone deacetylation, as would occur for any integrated transgene. It has been shown that silenced transgenes can be reactivated using histone deacetylase inhibitors such as butyrate or trichostatin A (Chen *et al.*, 1997; Pikaart *et al.* 1998). Therefore, adding one such inhibitor to the cell growth medium may allow re-activation of the promoter and expression of the MLTU.

7.2.3. Defects in RNA processing and translation.

There is a possibility that the problem lies not with transcription of the MLTU but with post-transcriptional processing or translation. These events are regulated at multiple levels during viral infection and involve the interaction of several viral proteins (section 1.3.5.4). The lack of these proteins in the cell line, even if provided by helper virus, may lead to a block in late protein expression.

The regulated RNA splicing of the MLTU is a very complicated process, with changes in splice site usage occurring as the infection proceeds from early to late phases. An example of this is the formation of L1 mRNAs, which moves from

generation of only the 52/55K mRNA at early times, to both the 52/55K and IIIa mRNAs at late times. This activation of IIIa splicing, late after infection, has been shown to be accompanied by a virus induced dephosphorylation of the cellular family of SR splicing factors (Kanopka *et al.*, 1998). This modification reduces the RNA binding capacity of SR proteins and as a consequence relieves the repression of IIIa 3' splice site usage, resulting in a shift toward an increase in IIIa mRNA production. Recent studies have also shown that overexpression of the SR protein ASF/SF2 prevents the early to late shift in late gene expression (Molin and Akusjarvi, 2000). In addition to regulating the switch from proximal to distal splice site usage, ASF/SF2 overexpression may directly interfere with transcription from the major late promoter. Many RNA processing factors, including ASF/SF2, have been shown to interact with the C-terminal domain tail of RNA polymerase II (reviewed by Bentley, 1999). Such an interaction may have, or induce, adverse effects on transcription initiation and/or elongation at the major late promoter.

The function of these SR factors in regulating gene expression could provide a possible explanation for the lack of observed late protein expression in the stable cell lines. During transient transfection the concentration of ASF/SF2 would be lower relative to the plasmid copy number, than it would be if the plasmid copy number was reduced during stable cell line selection. Thus, during transient transfection, ASF/SF2 might be unable to repress the use of distal 3' splice sites and the full pattern of late mRNAs would be produced, leading to the observed expression of a wide range of late proteins. However, if plasmid copy number is low, the relatively higher concentration of ASF/SF2 would block the temporal shift in pre-mRNA splicing. If this is the situation in the stable cell lines, they might be expected to express the L1 52/55K protein. This protein was undetectable by Western blotting but expression could be so low, because of the effects on promoter activity already discussed, as to be undetectable. Of course, if ASF/SF2 acts to directly repress transcription then no expression of late proteins would be expected, as was the case in the results presented here.

If time had allowed, experiments would have been designed and carried out to determine if the primary RNA transcript was being correctly spliced. This could have been achieved by using Northern blot analysis to detect late mRNAs in total cellular RNA extracts. Short oligonucleotide probes, specific for the 5' ends of the late mRNAs, could be used to determine if any transcription from the MLP was occurring.

If the probes were to detect the mRNA species of the predicted length, then it could be assumed that transcription and splicing were occurring normally. If the mRNA species were larger than predicted then there must be a problem involving incorrect processing of the MLTU primary transcript. A total lack of late mRNAs would point back to a problem with transcription initiation, possibly caused by methylation or histone deacetylation as discussed in section 7.2.2.

If the stable cell lines were shown to be actively transcribing the MLTU, experiments could be designed to determine if the level of late protein expression was so low as to be undetectable by biochemical assays, but was enough to complement late gene deficiencies. This could be achieved by assessing the ability of the cell lines to complement the growth of appropriate temperature-sensitive (ts) mutants at the non-permissive temperature. The biochemical assays used may not be sensitive enough to detect low levels of late protein in the cells but this low level of expression may be enough to complement growth. Therefore, the use of a biological assay may be more appropriate as the intended use of the cell lines is to complement the growth of vectors with late gene deficiencies.

7.3. Down-regulation of the sMT-Ia promoter.

The results from the work carried out using the sMT-Ia/MLTU construct (Chapter 6) mirrored the problems encountered with the original MLP/MLTU construct. This plasmid was also capable of expression in a transient assay but not when present in a stable cell line. The sMT-Ia promoter could be induced successfully 24 hours after transfection, but not after selection and isolation of transfected cells. This promoter has been widely used to express heterologous genes in eukaryotic cells and its apparent non-functionality here was unexpected. The sMT-Ia promoter does contain several CpG dinucleotides, some of which are present in the metal response element (MRE) binding sequences. Methylation of these sequences could lead to down regulation of the promoter for the reasons discussed above (section 7.2.2).

The inability of the SMT-Ia promoter to drive the expression of late proteins suggests that it is some intrinsic property of the MLTU that is preventing the isolation of late protein-expressing cell lines. Due to time limit restrictions, further evaluation of the sMT-Ia promoter was not possible. The use of a control plasmid containing the

EBV sequences and a reporter gene driven by the sMT-Ia promoter could have determined whether the promoter was capable of regulating gene expression during long-term maintenance of the plasmid in a 293-derived cell line.

7.4. Strategies to allow stable expression of late proteins.

The initial lack of expression in the 293-EP stable cell line was assumed to be due to inactivation of the MLP, perhaps due to selective pressure to reduce toxic protein expression. For this reason the MLP was replaced by the sMT-Ia promoter in an attempt to achieve tightly regulated expression, thus preventing potentially toxic basal levels of late protein expression. This approach was also unsuccessful with possible reasons discussed above (section 7.3). In addition to these problems, late protein expression from the sMT-Ia/MLTU construct was detected shortly after transfection in the absence of the inducing agent (section 6.4.3). This again could have led to promoter down-regulation to prevent the expression of toxic proteins. Any future attempts to express the MLTU in 293 cells should use a more tightly regulated promoter, such as the tetracycline-regulated promoter, in order to prevent this potentially deleterious basal expression. Such a system could be implemented by placing the MLTU under the control of a Tet-responsive (Gossem et al. 1995), bi-directional promoter (TRBDP), which should enable tight control of gene expression and prevent any down-regulation of gene expression due to toxic protein expression. The construct will also express the GFP reporter gene, enabling regulated expression of both this and the MLTU in a stable cell line. The presence of the GFP gene will allow easier monitoring of the transcriptional activity of the construct.

Such a cell line is currently being developed (D.Farley, personal communication) and will be created by selecting for integration of the TRBDP / MLTU / GFP cassette (co-transfected with a plasmid containing a selectable marker gene) into HeLa cells using antibiotic resistance. Clones of cells will be isolated and grown in the presence of tetracycline (or doxycycline) which will repress transcription from the TRBDP, a so-called “Tet-off” system (Gossen and Bujard, 1992). In this system, tetracycline inactivates the transcriptional-transactivator ‘reverse tTA’ (provided in trans by the cell) such that the promoter is inactive and no expression of the MLTU or GFP should occur. If tetracycline is removed from the growth medium the promoter is activated

and transcription of the MLTU and GFP should now occur. Clones can be quickly screened using a GFP assay that will indicate successful integration of the expression cassette into the cellular genome at locations that allow functionality of the promoter. GFP-positive clones would therefore indicate potential expression of late proteins from the MLTU, which could then be characterised using the appropriate assays.

In addition to using alternative promoters, another way to express late proteins in stable cell lines could be to split the MLTU and express the late genes as two separate units from two expression plasmids. Through the use of two selectable markers a double-stable cell line could be created expressing the full range of late genes. Earlier manipulation of the MLTU to replace the MLP (Chapter 6) suggests that carefully considered changes can be made which still allow the MLTU to be expressed correctly. Therefore, it is possible that the MLP or an inducible promoter could be cloned upstream of any late gene and used to drive its expression as long as the tripartite leader was also included to enable efficient translation of the viral mRNA.

If the lack of late protein expression is due to the activity of SR factors, then strategies could be devised to reduce their activity and allow correct transcription and processing of the MLTU. The activity of SR proteins is reduced by dephosphorylation mediated by protein phosphatase 2A (PP2A, Kanopka *et al.*, 1998). The adenovirus E4-orf4 protein has been shown to bind to PP2A and down-regulate the transcription of the cellular gene *junB* (Kleinberger and Shenk, 1993). In addition to this the work of Kanopka *et al.*, (1998) also suggests that E4-Orf4 is an important factor in controlling the temporal shift in adenovirus alternative RNA splicing. If this is the case then constitutive expression of E4-Orf4 may allow the expression of late proteins in a stable cell line.

7.5. Alternative uses for Ad5 expression plasmids.

In addition to their use in the construction of cell lines to complement adenovirus vectors, the expression plasmids constructed here also have other potential uses in the study of late protein expression. In transient transfection, the plasmids apparently express proteins from across the length of the MLTU. During infection, this pattern changes with time due to regulated RNA processing as discussed above. The plasmid could be used to introduce mutations into MLTU splice sites in order to

study the effects on splicing patterns and the effect on expression of upstream and downstream reading frames.

A late protein-expressing cell line could also be used to study the late proteins that have poorly understood functions, such as the L4-33K and L4-100K proteins. There is absolutely no functional information about L4-33K, while the function of the L4-100K protein has so far only been studied by using temperature sensitive (*ts*) mutants, which work has implicated it in viral particle assembly (Oosterom-Dragon and Ginsberg, 1981) and translational control (Adam and Dreyfuss, 1987; Hayes *et al.*, 1990). L4-100K may also be involved in directly inhibiting cellular protein synthesis and in enhancing expression of viral late mRNAs (reviewed by Schneider, 2000). The ability to create further deletions and mutations in the L4-100K protein should lead to further understanding of its functions.

7.6. Concluding remarks.

Human adenoviruses have been extensively studied and characterised since their initial isolation in the 1950's (Hilleman and Werner, 1954; Rowe *et al.*, 1953). Despite this extensive research there are still many aspects of the viral replication cycle that are poorly understood, particularly aspects of virus–cell interactions and the host immune response to infection. In the three years during which the research in this thesis was carried out, over 4,000 references to adenovirus were published. Most of these were concerned with the results of investigations using adenoviruses as vectors and relatively few dealt with the basic virology and immunology of virus infection. These shortcomings in the understanding of adenovirus biology have become more apparent with the increasing use of adenovirus vectors in gene therapy protocols. It is now accepted that the initial enthusiasm for utilising adenovirus vectors in clinical gene therapy trials was rather prematurely optimistic and perhaps over-hyped. Serious problems associated with immune responses to the vector have led to the promised potential of adenovirus vectors being largely unrealised.

The work presented in this thesis aimed to improve adenovirus vector technology by allowing further engineering of the genome to reduce late protein expression. Due to an inability to isolate a stable cell line that would express late proteins, these initial aims were not achieved. This was partly due to a poor

understanding of how the MLTU is expressed when taken out of the context of a natural viral infection. Further understanding of late gene expression may allow the isolation of vectors containing large deletions that would express fewer viral proteins and should therefore provoke a less vigorous immune response *in vivo*, leading to increased persistence. Given that no one has, as yet, been able to show very long-term persistence of expression from genes transduced by adenovirus vectors, this remains a valid approach for further research into vector construction.

Adenovirus vectors in current use have proved to be largely ineffective, but a better understanding of the total spectrum of virus-cell and virus-host interactions should lead to the design of vectors providing more efficient delivery along with minimal deleterious host reactions. As a result of continued research, the problems associated with current adenovirus vectors may well be overcome and the desired clinical goals achieved.

Chapter 8 : Bibliography.

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Appendix.

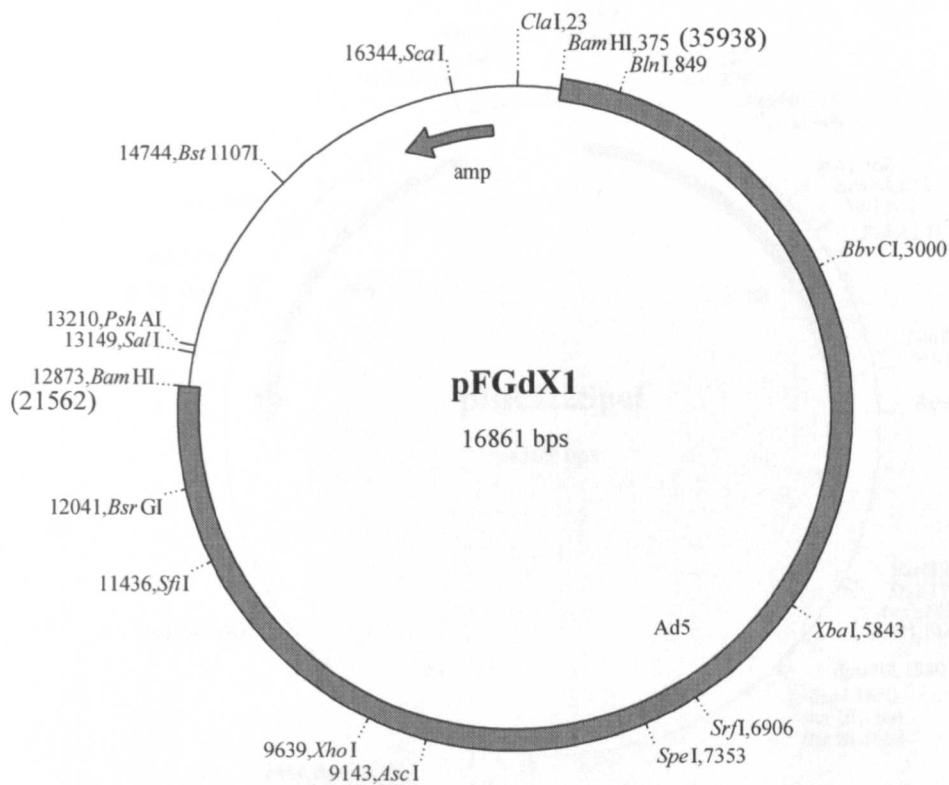


Figure A.1. Plasmid pFGdX1.

pBR322-based plasmid bearing Ad5 sequences from 21562-35938, with a deletion in the E3 region between bp 28592 and 30470 . Map shows unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). amp, ampicillin resistance gene.

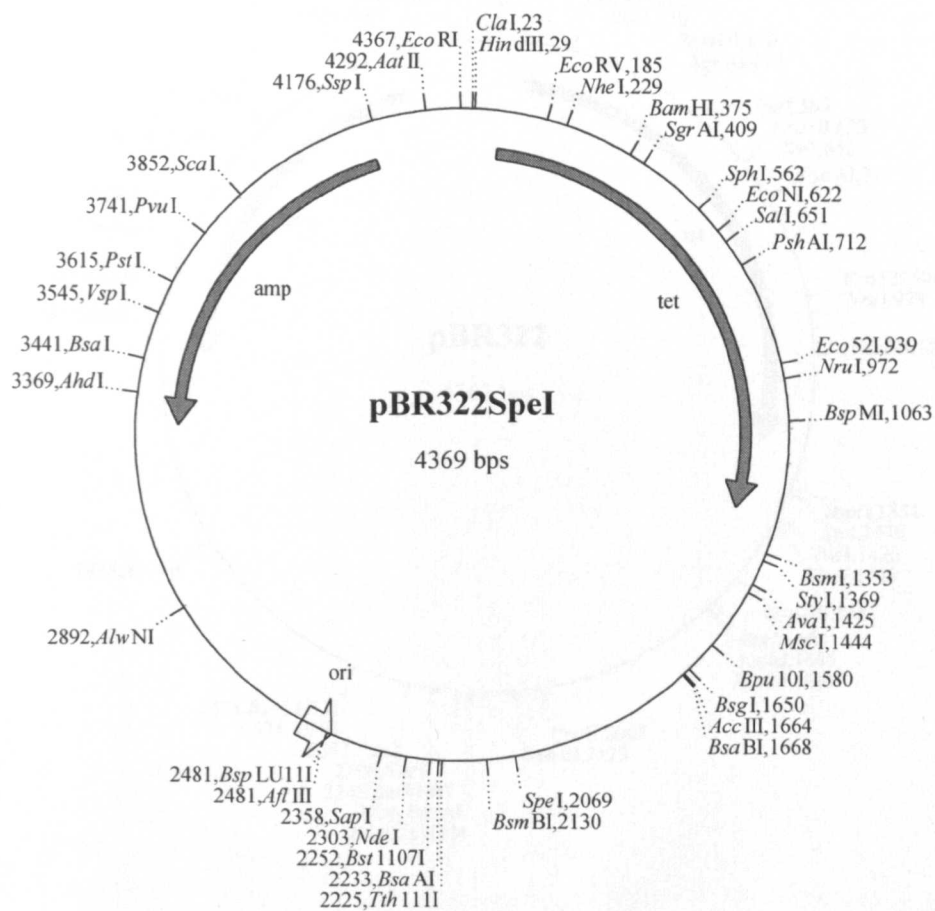


Figure A.2. Plasmid pBR322SpeI.

pBR322 with *Pvu*II at bp 2064 converted to *Spe*I. Restriction map shows unique sites. amp, ampicillin resistance gene; tet, tetracycline resistance gene.

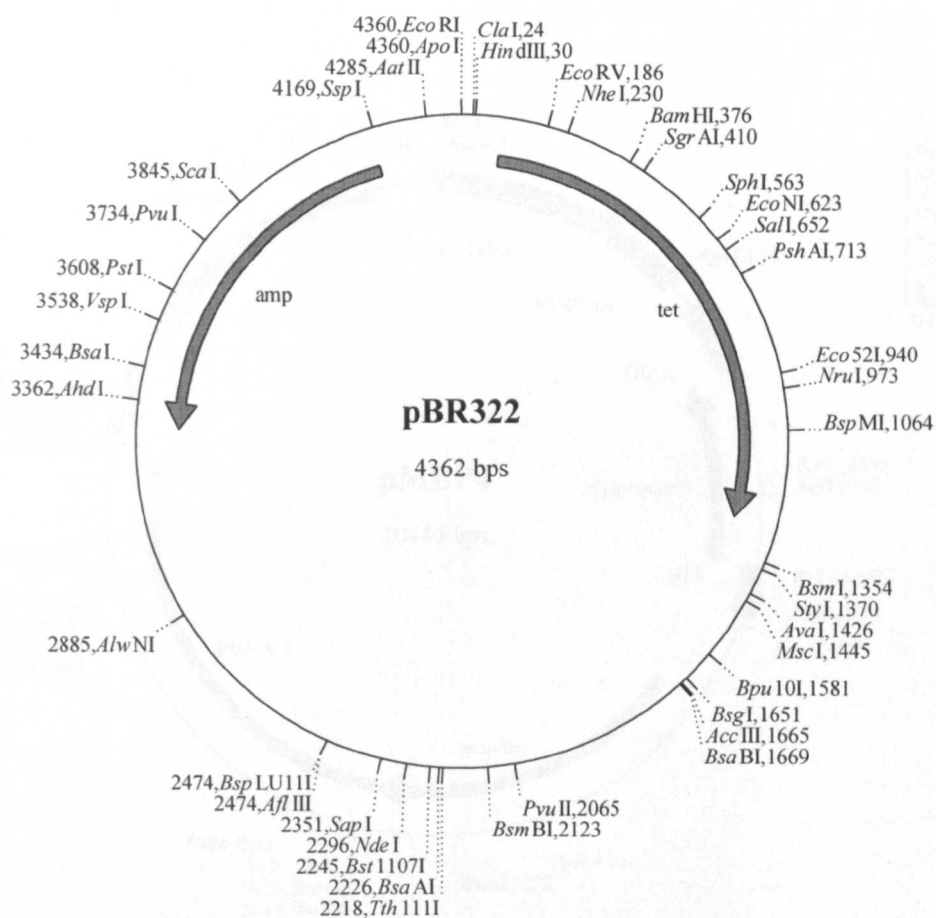


Figure A.3. Plasmid pBR322.

Restriction map shows unique sites. amp, ampicillin resistance gene; tet, tetracycline resistance gene.

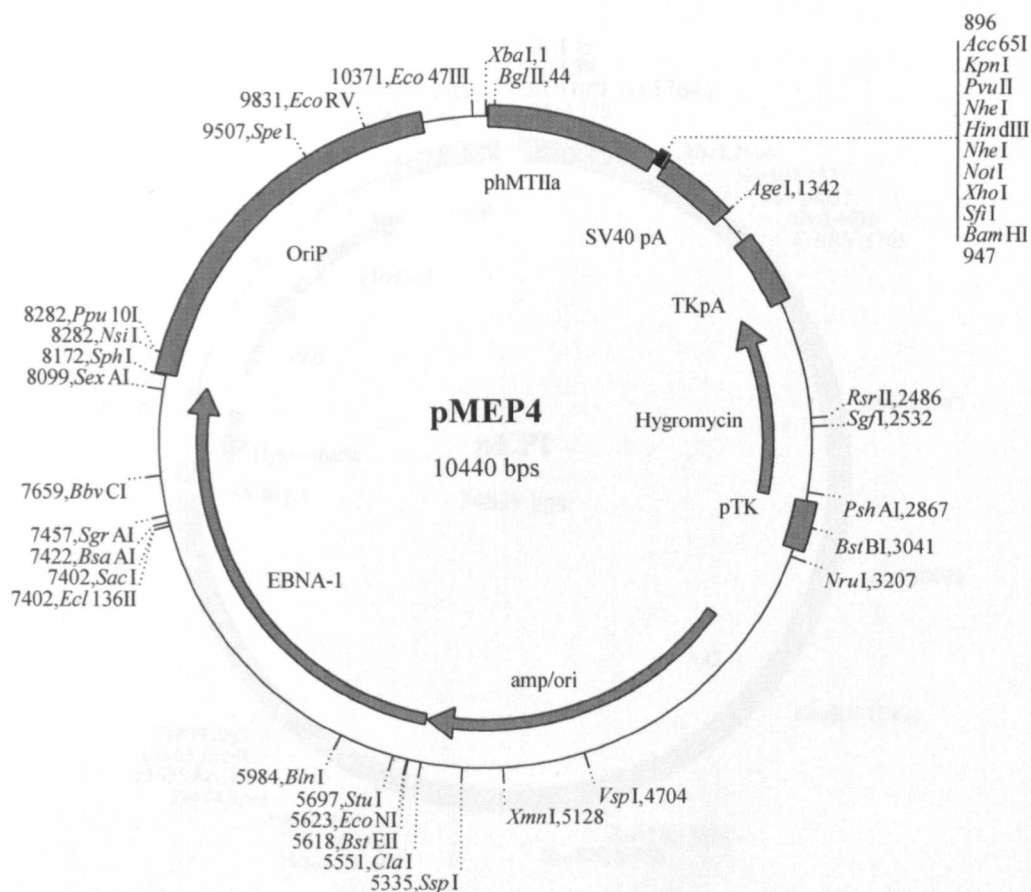


Figure A.4. Plasmid pMEP4 (Invitrogen).

Map showing unique sites and sites of interest. Abbreviations: pTK, thymidine kinase promoter; Hygromycin, hygromycin-B resistance gene. TKpA, thymidine kinase polyadenylation signal; SV40 pA, simian virus 40 polyadenylation signal; phMTIIa, human metallothionein IIa promoter; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp/ori, ampicillin resistance gene/bacterial origin of replication.

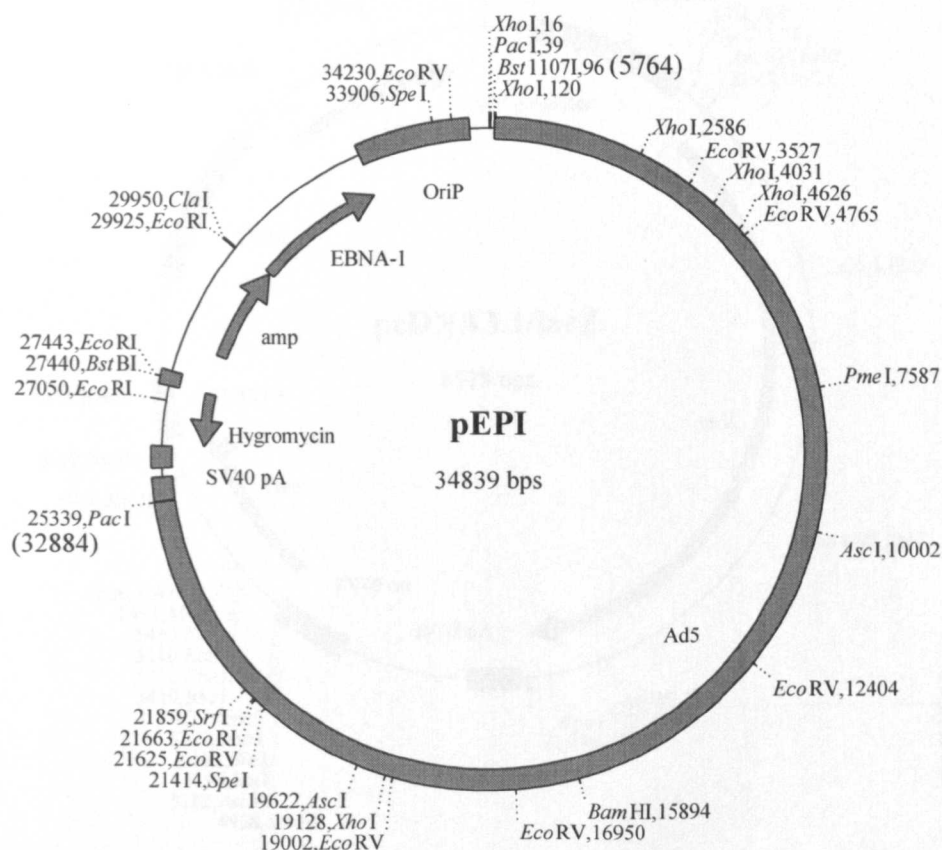


Figure A.5. Plasmid pEPI.

Plasmid map showing unique sites and sites of interest. Numbering in brackets refers to original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). Abbreviations: Hygromycin, hygromycin-B resistance gene; SV40 pA, simian virus 40 polyadenylation signal; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene; Ad5, adenovirus sequence.

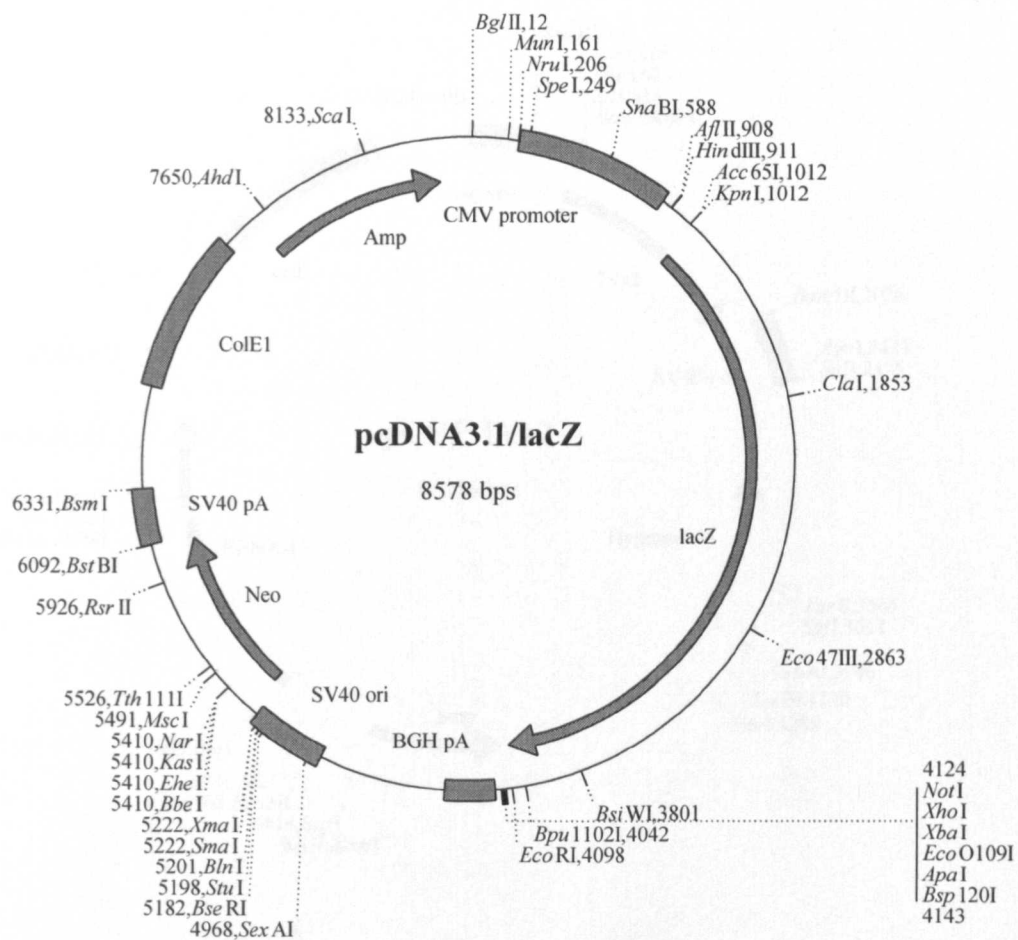


Figure A.6. Plasmid pcDNA3.1/lacZ.

Plasmid containing *lacZ* reporter gene. Map showing unique sites. Abbreviations: amp, ampicillin resistance gene; neo, neomycin resistance gene; CMV promoter, cytomegalovirus immediate early promoter.

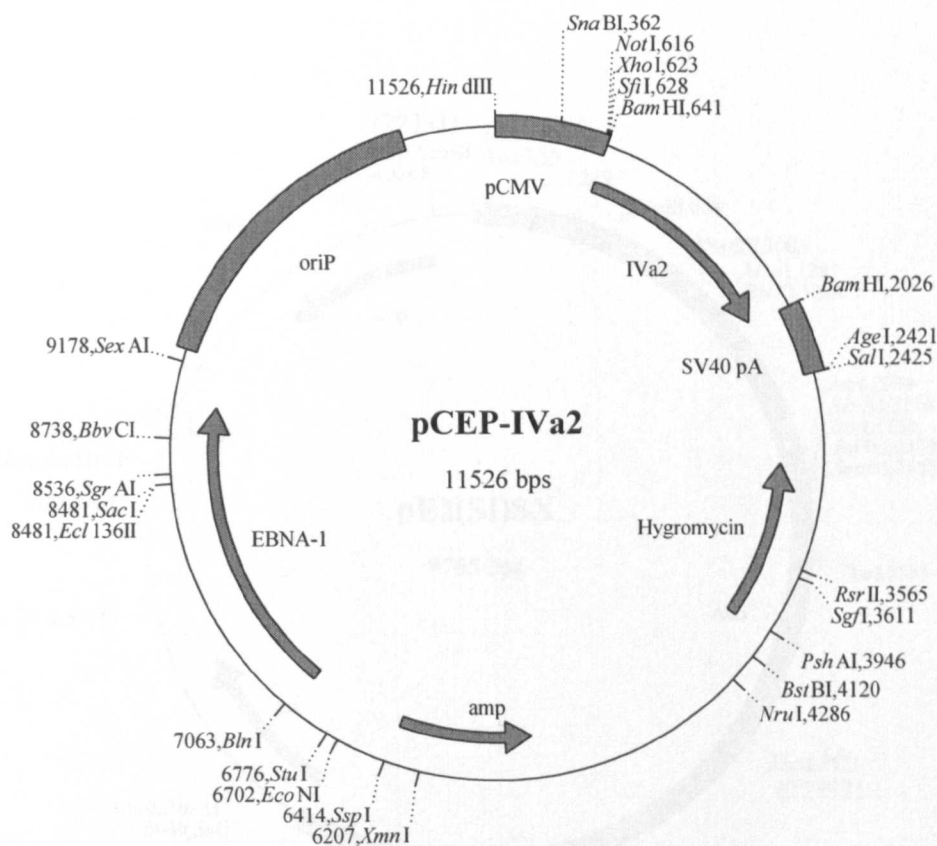


Figure A.7. Plasmid pCEP-IVa2. Episomal expression plasmid containing the IVa2 cDNA from Ad5. Abbreviations: Hygromycin, hygromycin resistance gene; SV40 pA, simian virus 40 polyadenylation signal; pCMV, human cytomegalovirus immediate early promoter; OriP, EBV latent origin of replication; EBNA-1, Epstein-Barr virus nuclear antigen 1; amp, ampicillin resistance gene.

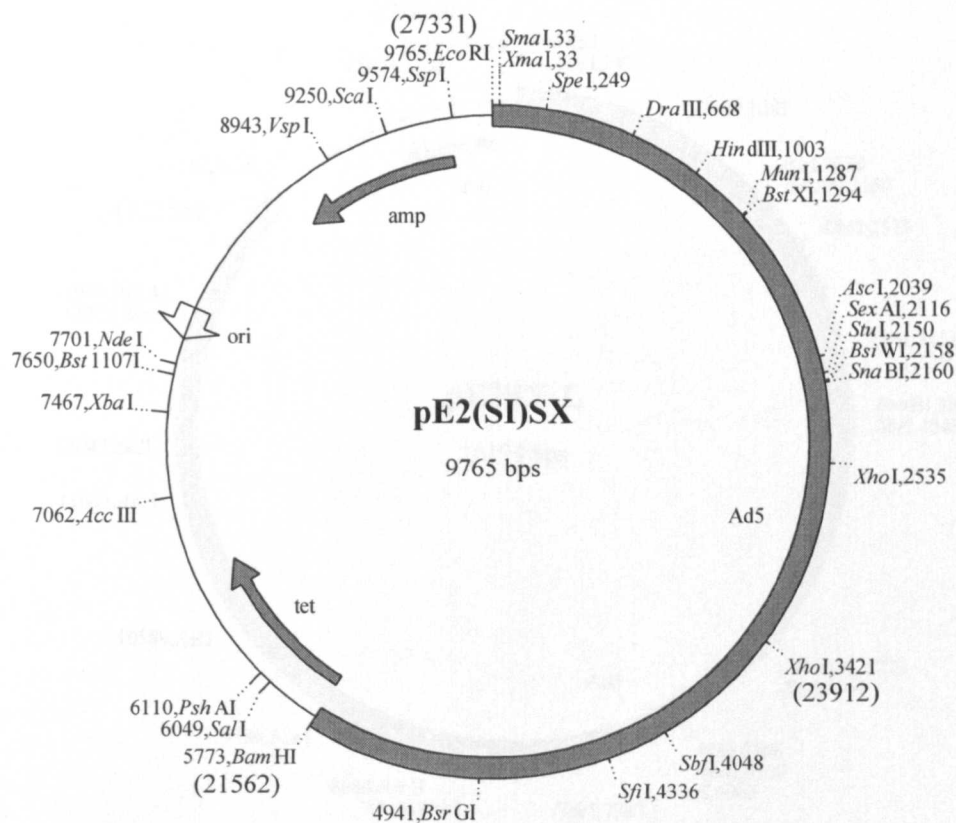


Figure A.8. Plasmid pE2(SI)SX.

pBR322-based plasmid bearing Ad5 sequence from 21562-27331. Contains *Sma*I to *Xho*I modification at bp pos. 23912. Map shows unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). amp, ampicillin resistance gene; tet, tetracycline resistance gene.

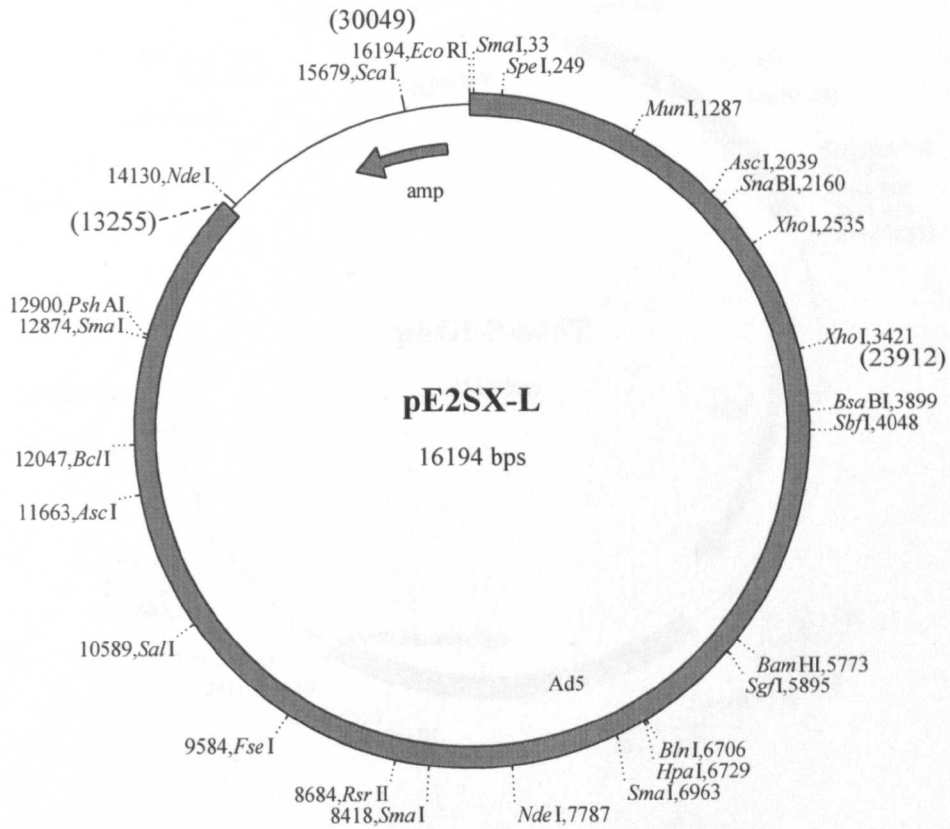


Figure A.9. Plasmid pE2SX-L.

pBR322-based plasmid bearing Ad5 sequence from 13255-27331. Contains *Sma*I to *Xho*I modification at bp pos. 23912. Map shows unique sites and sites of interest. Numbering in brackets refers to the original numbering in the Ad5 sequence (Chroboczek *et al.*, 1992). amp, ampicillin resistance gene.

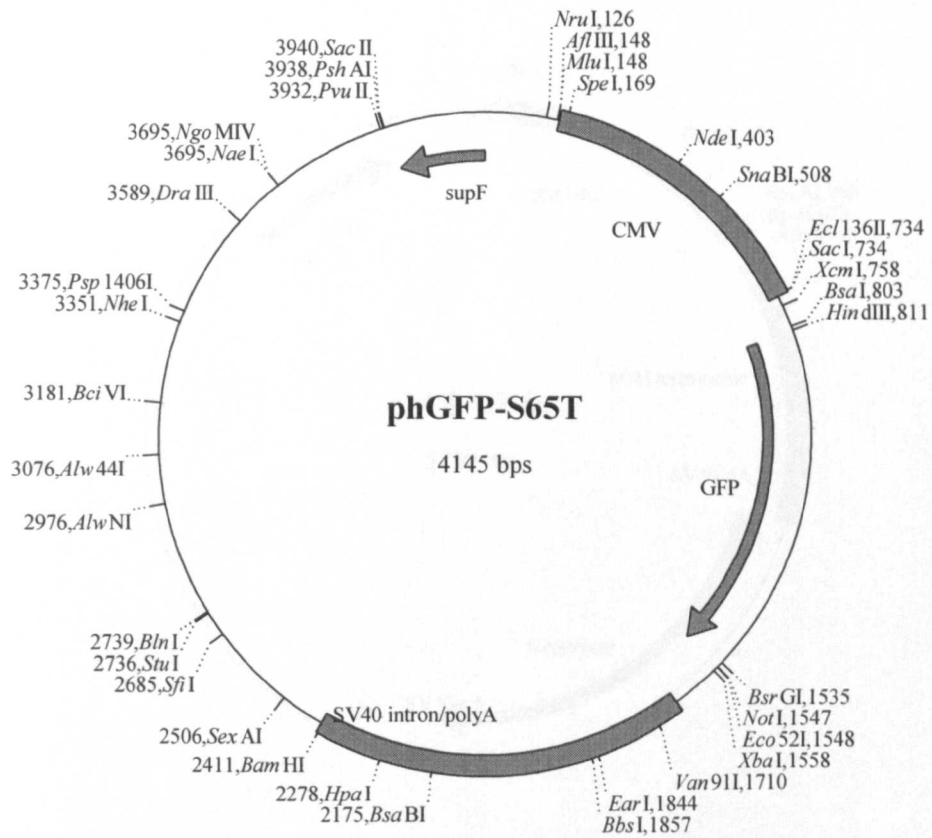


Figure A.10. Plasmid phGFP-S65T.

Green fluorescent protein expression plasmid. Restriction map shows unique sites. Abbreviations: CMV, cytomegalovirus immediate-early promoter; GFP, green fluorescent protein; SV40 intron/polyA, simian virus 40 intron and polyadenylation sequences; *supF*, ampicillin and tetracycline resistance gene.

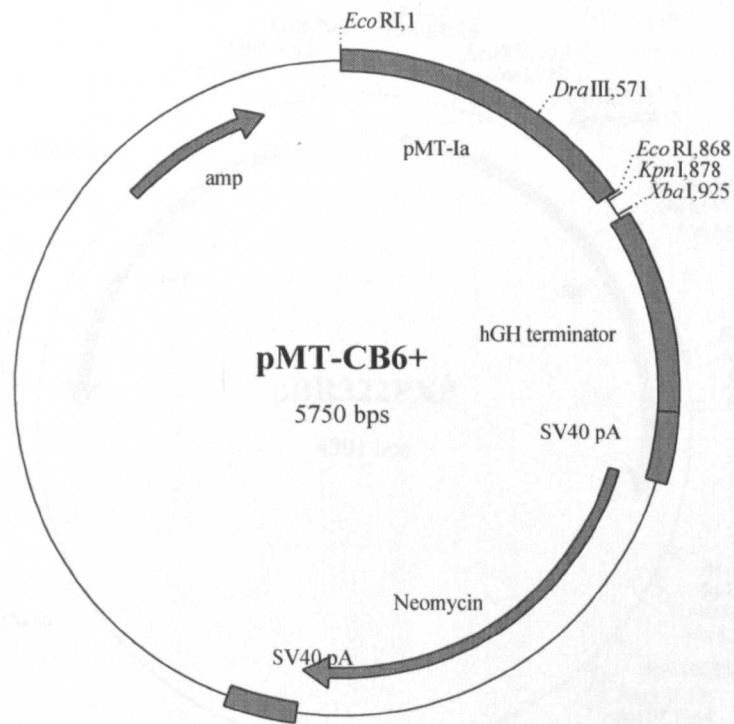


Figure A.11. Plasmid pMT-CB6+. Expression vector containing the sheep metallothionein-Ia promoter. Amp, ampicillin resistance gene; neomycin, neomycin resistance gene; pMT-Ia, sheep metallothionein promoter.

